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Development of postsynaptic function in muscle membrane

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DEVELOPMENT OF POSTSYNAPTIC FUNCTION IN MUSCLE MEMBRANE

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DEVELOPMENT OF POSTSYNAPTIC FUNCTION IN MUSCLE MEMBRANE

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ABSTRACT

The development of postsynaptic function in skeletal muscle of *Xenopus laevis* was studied *in vivo* in order to address the following questions: What changes take place in acetylcholine receptor (AChR) channel function during muscle development and when do they occur? Does muscle activity regulate the development of postsynaptic function? Do functionally different muscles have different programs of postsynaptic development?

Single channel recordings from nonjunctional membrane revealed a class of low conductance (30 to 40 pS), long open time (2-3 ms) AChR channels which appeared on embryonic membrane within 21 h of fertilization. At 45 h of age, a second class of higher conductance (40 to 60 pS), brief open time (<1 ms) channels began to be expressed and over the course of 4 days became the most frequently observed channel type. Concurrently, the open time of the low conductance channel decreased by half during development. These data explain the developmental change in duration of synaptic currents previously observed in myotomal muscle, and they lay the foundation for further studies on the molecular mechanisms of AChR development.

The effect of immobilization on the development of synaptic currents in myotomal muscle was investigated by allowing embryos to develop in a bath containing tetrodotoxin, which eliminated muscle activity during formation and maturation of the neuromuscular junction. In both control and tetrodotoxin-immobilized animals, synaptic current rise times and decays developed in an equivalent fashion, indicating that muscle activity is not required for normal development of AChR channel gating or acetylcholinesterase (AChE) deposition at the neuromuscular junction.

The development of synaptic currents was compared in two functionally different muscles, the interhyoideus and the superior oblique. Each muscle has a characteristic program of synaptic current development during synaptogenesis and during metamorphosis. The contrasting development of synaptic currents from the two muscles can be explained by different programs of AChR and AChE development.

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ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
AChR	acetylcholine receptor
BTX	bungarotoxin
ChAT	choline acetyltransferase
EPC	endplate current
EPP	endplate potential
MEPC	miniature endplate current
MEPP	miniature endplate potential
MSF	methanesulphonyl fluoride
NMJ	neuromuscular junction
TTX	tetrodotoxin

Standard SI abbreviations are used for all physical units.

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INTRODUCTION

The ability of the nervous system to process information depends on the establishment of orderly connections, or synapses, between its different cellular components: neurons, receptors and effectors. These connections arise largely during the early development of the nervous system. Some synaptic connections are genetically determined, while others are susceptible to environmental influences. The principles governing synapse development during embryonic development may also apply to recovery after injury, and perhaps learning and memory. Understanding the principles of synaptogenesis is one of the major challenges of developmental neurobiology.

Although there is understandably strong motivation to study the development of synapses within the central nervous system, this has proven to be difficult due to the immense complexity of CNS synaptic connections and their lack of accessibility to physiological recording instruments. Most information about synapse structure, function, and development comes from the vertebrate neuromuscular junction (NMJ). Because of the relative ease in obtaining data from the NMJ, a wealth of information has accumulated about its structure and function in both mature and developing muscle. The NMJ is often used as a model synapse with respect to synaptogenesis. Although synapse development in other parts of the nervous system is now being studied, there is, as yet, no compelling reason to believe that synaptogenesis elsewhere is fundamentally different from that at the NMJ.

The development of the NMJ has been examined in a limited number of vertebrate systems. Most of what is presently known derives from studies of rat, chick and frog. A major consensus from this work is that the synapse development occurs as an orderly series of events with a predictable schedule. The development of the NMJ appears to be carefully regulated with respect to both the nature and timing of its construction.

The NMJ begins to mediate neurotransmission long before it is either structurally or functionally mature. Most of the previous work describing the changes in function during development has been conducted with embryonic neurons and muscle cells in culture. Valuable information has been derived from these studies about development of both pre- and postsynaptic function. However, a major drawback to *in vitro* studies of synapse development is that the cells develop in an artificial environment, isolated from their normal embryonic millieu. The extent to which synapse development between cultured cells reflects normal development *in vivo* can only be determined by complementary studies in the intact animal.

With this in mind we have examined in detail the development of postsynaptic function in *Xenopus laevis* (the South African clawed frog). The aim of this work was to address the following questions regarding NMJ development:

1. What changes in acetylcholine receptor function occur during muscle development *in vivo* and when do they take place?

2. Are action potentials and contractile activity of muscle necessary for the normal development of postsynaptic function?

3. Do muscles which possess different functional characteristics also have different programs of synapse development?

These questions could only be appropriately answered through examination of normally developing muscle in the intact animal. Considerable knowledge already exists about the neuromuscular junction. In order to better understand the relevance of the results presented in this thesis, background information describing the structure, function, and development of the neuromuscular junction will be presented.

BACKGROUND

Structure of the Neuromuscular Junction

The structure of the frog NMJ has been well described and serves as a model for both peripheral and central chemical synapses. The NMJ was first described at the light microscope level in the 1840's (reviewed by Salpeter, 1987a), but its detailed structure was clarified only after the advent of transmission electron microscopy about a century later.

The NMJs of adult frogs exhibit several regular features (Salpeter, 1987a). Where it contacts the muscle, the axon repeatedly branches into terminal projections as long as 300 μm which reside in narrow (1-3 μm wide) gutters or troughs on the muscle membrane. The myelin sheath terminates before the axon branches, but the terminal axon branches are covered by Schwann cells. The Schwann cells which cover the nerve terminal send fingerlike processes around it which divide the synaptic cleft into regularly spaced compartments.

When thin sections of the NMJ are viewed with the transmission electron microscope, specific ultrastructural details are consistently observed. Typically, no ribosomes or rough endoplasmic reticulum are observed in the nerve terminal; however, smooth endoplasmic reticulum is present and may serve as an intracellular calcium store (Blaustein *et al.*, 1980). The nerve terminal also contains agranular synaptic vesicles, which are clustered about narrow electron dense bars, or active zones, spaced about one μm apart. These vesicles are believed to contain acetylcholinesterase (AChE) (Heuser *et al.*, 1979). Freeze fracture replicas of presynaptic membranes, examined with the scanning electron microscope reveal double rows of particles paralleling each active zone. These particles are thought to be voltage gated calcium channels, which may mediate exocytosis by permitting an influx of calcium during depolarization of the presynaptic membrane (Heuser *et al.*, 1974; Heuser *et al.*, 1979; Pumplin *et al.*, 1981; Walrond & Reese, 1985). Lipid composition of the active zone membrane may especially favor vesicle fusion and release. This region of the presynaptic membrane is known to be low in cholesterol (Nakajima & Bridgeman, 1981; Ko & Probst, 1986) which probably affects its fluidity. A gap of about 50 nm, the synaptic cleft, separates the pre- and postsynaptic membranes. The cleft is continuous with the extracellular space and is partially filled with an extracellular matrix, or basal lamina, composed of collagen, proteoglycans, elastin, and glycoproteins, especially fibronectin and laminin (Hay, 1983). The synaptic basal lamina is part of the matrix material which envelops the muscle and Schwann cells. It follows the contour of the postsynaptic membrane, which is sometimes quite convoluted. It is now clear that the synaptic basal lamina is antigenically distinct (reviewed by Burden, 1987). It is important in directing reinnervation after denervation

and may be involved in organizing the postsynaptic membrane during NMJ development. AChE is anchored to the synaptic basal lamina and is present at a density of two to three thousand ACh binding sites per square μm (Salpeter *et al.*, 1972, 1978). It extends all the way to the bottom of the folds in the postsynaptic membrane.

The postsynaptic membrane of the neuromuscular junction is often extensively folded in twitch muscle. There are, however, important exceptions to this generalization. The postsynaptic membrane in *Xenopus* myotomal muscle is characterized by very shallow junctional folding (Kullberg *et al.*, 1977). There also may be considerable variation in junctional folding within twitch muscle of the same animal. For instance, the extraocular muscle of *Xenopus* tadpoles, in contrast to myotomal muscle, has deep and numerous junctional folds (Owens, unpublished data). Folds are typically spaced at regular intervals and are directly opposed to the presynaptic membrane between active zones. In amphibians the depth of the folds may reach 0.5 μm and folding can increase the surface area of the postsynaptic membrane several fold (Matthews-Bellinger & Salpeter, 1978). The functional significance of junctional folding is unknown. The folds may permit increased quantities of AChE at the synapse since the basal lamina to which it is anchored follows the contours of the postsynaptic membrane (Couteux, 1963; Salpeter, 1969).

The membrane of the junctional folds is heterogeneous. The electron microscope reveals a thickened region at the top of the folds which extends to about 50% of their depth in amphibians. The lower region of the fold often contains vesicles, both coated and non-coated (Hirokawa & Heuser, 1982). Freeze etch studies reveal arrays of densely packed particles residing in the membrane at the tops of the junctional folds (Peper *et al.*, 1974; Rash & Ellisman, 1974). The particle density is about 10,000 per square μm (Hirokawa & Heuser, 1982) and tapers off dramatically within a few μm of the synapse to negligible densities (Miledi, 1960; Kuffler & Yoshikami, 1975; Fertuck & Salpeter, 1974; Bekoff & Betz, 1977). Radiolabeled α-bungarotoxin (α-BTX), which specifically binds to AChRs (Lee, 1979), also binds to the tops of the junctional folds (Fertuck & Salpeter, 1974). It is now generally believed that the thickening at the tops of the junctional folds is due to accumulation of protein, especially AChRs and other molecules which may be associated with their anchoring or function.

Ion channels other than AChRs, are also differentially segregated at or near the NMJ. There is an increased concentration of voltage gated sodium channels (Betz *et al.*, 1984b; Beam *et al.*, 1985) and inward rectifying potassium channels (Katz & Miledi, 1982), in the vicinity of the NMJ but fewer chloride channels (Betz *et al.*, 1984a). This spatial distribution of ion channels probably increases the efficiency of the postsynaptic response to the release of ACh from the nerve terminal.

Neuromuscular Transmission

Our understanding of the main principles of neuromuscular transmission has evolved through the work of numerous investigators during the past four decades. Before considering some of the details of neurotransmission, the main events are summarized as follows. When an action potential depolarizes the nerve terminal, voltage gated calcium channels open and allow a transient influx of calcium. The increased concentration of free intracellular calcium facilitates the release of ACh from the terminal through mechanisms which are not well understood. It is generally accepted that ACh is contained within synaptic vesicles, and calcium increases the rate of vesicle fusion and exocytosis. Once ACh is released into the synaptic cleft, each molecule is subject to one of three fates. It may (1) diffuse out of the cleft, (2) be hydrolyzed by AChE, or (3) bind to AChRs. When the latter occurs, ion channels within the receptor proteins open, permitting the passage of small cations (Na^+ and K^+) across the muscle membrane. This ionic current results in depolarization of the endplate region. If the postsynaptic membrane is sufficiently depolarized, then the events leading to muscle contraction ensue.

Synthesis and packaging of acetylcholine

Acetylcholine is synthesized within the nerve terminal by the enzyme choline acetyl transferase (ChAT). Choline is acetylated by acetyl CoA in a simple, one step mechanism (for review of presynaptic function see Bradford, 1986; Jones, 1987). Choline is present in the extracellular space as a result of ACh hydrolysis and phospholipid turnover. It is also produced in the liver and delivered to other parts of the body via the circulation. Choline is taken up into cholinergic nerve terminals by both a high and low affinity pump. ACh is synthesized in the cytoplasm and subsequently loaded into vesicles. From physiological experiments (Kuffler and Yoshikami, 1975; Katz & Miledi, 1979), the number of ACh molecules contained in a single synaptic vesicle is estimated to be 1000 to 10,000. When packed into vesicles with an interior diameter of about 40 nm, the ACh concentration would vary from about 70 mM to 500 mM. There is evidence that active uptake of ACh into synaptic vesicles is linked to a proton gradient generated by a proton dependent ATPase (Harlos, *et al.*, 1984).

Release of acetylcholine

The most widely accepted concept of the events leading to transmitter release involves fusion of synaptic vesicles near the presynaptic active zone and exocytosis of their contents into the synaptic cleft. The molecular mechanisms involved in release are still being actively investigated and there is little agreement, except that calcium influx is probably just an initial step in a rapid sequence of events leading to exocytosis of ACh.

The vesicular release theory is supported by both morphological and physiological investigations, but there is also some evidence for the release of transmitter directly from the cytoplasm of mature (Isreal, 1979; Tauc, 1982) and embryonic nerve terminals (Sun & Poo, 1985). As a test of the vesicular release hypothesis, ingenious experiments were done in which the nerve terminal was instantaneously (< 10 ms) frozen during evoked neurotransmission (Heuser *et al.*, 1979) then examined with transmission electron microscopy. These experiments revealed flask-like invaginations of the presynaptic membrane or "omega" profiles opening into the cleft. The profiles were interpreted as synaptic vesicles caught in the act of expelling their contents. Further support for vesicular release of ACh came when the electron microscope revealed that intensely stimulated nerves contained a diminished number of synaptic vesicles (Zimmerman *et al.*, 1977). Following exocytosis the vesicle membrane is probably recycled via coated pits from the presynaptic plasma membrane (Heuser & Reese, 1973; Caccarelli *et al.*, 1979; Miller & Heuser, 1984).

Electrical recordings of postsynaptic activity also suggest that transmitter is released in discrete units or "quanta". Each quantum or packet of transmitter is believed to give rise to a small postsynaptic current known as a miniature endplate current (MEPC). MEPCs can be recorded by placing an extracellular electrode over the postjunctional membrane or by voltage clamping the postjunctional membrane. The change in voltage associated with the MEPC is called a miniature endplate potential (MEPP). MEPCs occur spontaneously, even when action potentials in the nerve are blocked, but usually do not depolarize the membrane sufficiently to initiate contraction of the muscle. However, when a nerve impulse arrives at the terminal, a large depolarization, or endplate potential (EPP), occurs. The EPP is usually large enough to trigger an action potential in the postsynaptic membrane, leading to muscle contraction. The relatively constant amplitude of MEPPs gave rise to the classical studies by del Castillo and Katz (1954) which demonstrated that EPPs are the summed result of hundreds of MEPPs. The concept that the EPP is composed of many MEPPs is known as the quantal theory of transmission. It is now well established that the number of individual packets of transmitter released during an EPP is directly related to the amount of calcium entering the cytoplasm of the terminal (Dodge & Rahamimoff, 1967; Llinas *et al.*, 1981a,b; Augustine *et al.*, 1985a,b).

Time course of endplate currents

MEPC waveforms have a rapid rising phase and an exponential decay. The shape and time course of the MEPC resembles the much larger endplate current (EPC), but MEPCs are more convenient to study because they do not cause muscle movement. In addition, the MEPC waveform is not distorted by electrical properties of the membrane, unlike intracellular voltage recordings of MEPPs or EPPs (del Castillo & Katz, 1956; Katz &

Miledi, 1973). Much of our present understanding of synapse function has resulted from the analysis of MEPC waveforms, which reflect all of the important spatial and kinetic parameters of synaptic transmission.

To account for the MEPC waveform, Salpeter and her collaborators (reviewed by Salpeter, 1987a) proposed the "saturated disc" model based on kinetic and anatomical measurements. This model predicts that ACh molecules are bound to receptors or else hydrolyzed by AChE before traveling more than about 0.3 μm from the point of release. Based on calculations from MEPC amplitudes and the single channel conductance of AChRs, it appears that 1000 to 2000 AChR channels are opened during an MEPC (Katz & Miledi, 1972; Anderson & Stevens, 1973). Two molecules of ACh must bind to the AChR before it will open (see review of endplate function by Adams, 1987). The concentration of ACh within the cleft is expected to exceed 0.5 mM at the time of release (Matthews-Bellinger & Salpeter, 1978). The high concentration of ACh results in a small saturated disc of doubly liganded receptors and fully bound AChE, surrounded by singly or unliganded receptors. There are about 5 fold more AChRs than AChE molecules at the junction and it is postulated that during the rising phase of the MEPC (about 0.1-0.2 ms), AChE will be saturated with ACh leaving the bulk of the transmitter to bind to receptors. To produce a rapidly rising MEPC, ACh has to bind rapidly to AChRs before it diffuses away, and the channels have to open rapidly in response to ligand binding. Both of these conditions are probably met. Land *et al.* (1984) estimated that the rate constant for ACh binding to the receptor is greater than 10^7 M^{-1} s^{-1} and the opening rate constant for the channels is about 25,000 s^{-1}.

It is thought that ACh unbinds from receptors only after they close. As ACh molecules unbind from AChRs they are probably hydrolyzed so rapidly by AChE that receptors are activated only once during the MEPC (Katz & Miledi, 1973). This is due to the exceptional hydrolytic efficiency of AChE, which has a turnover rate of about 0.1 ms, (Lawler, 1961) as compared to a mean AChR channel open time of 1-3 ms (in *Xenopus*). These kinetic data indicate that the hydrolysis of ACh by AChE should easily keep up with the rate of unbinding of transmitter from receptors.

Magleby and Stevens (1972a) described the decay of endplate currents as a simple exponential function with a time constant, τ, which is the time required for the endplate current to decline e-fold. The rate constant of the exponential decay, α, was defined as the reciprocal of the time constant ($\alpha=1/\tau$). They considered two possible explanations for the exponential decay of endplate currents (Magleby & Stevens, 1972b). The first was that the channel closing rate was much faster than the disappearance of transmitter from the cleft, and ACh concentration in the cleft declined exponentially with a rate

constant of α. This explanation was discounted because the decay phase of the EPC was voltage dependent and exhibited a much higher dependence on temperature than would be expected for diffusion.

The second possible explanation was that ACh was removed very rapidly from the cleft and the rate of channel closing determined the rate of EPC decay. This explanation was consistent with their temperature and voltage clamp experiments and was further supported by Anderson and Stevens' (1973) study of AChR gating kinetics. They analyzed the spectral characteristics of the current noise observed during a steady application of ACh to the membrane, using the methods developed by Katz and Miledi (1970). They found that the noise spectrum could be well described by a Lorentzian function and gave a predicted channel closing rate which was equal to the rate of EPC decay. These studies led to the conclusion that the mean open time of the endplate channels could be estimated directly from synaptic current decay. This concept has proven very useful in understanding endplate physiology. Much of what we know about endplate channel gating and development of AChE activity at the NMJ has come from measuring the decay of synaptic currents.

The assumption that the decay phase of EPCs directly reflects channel closing can only be true if ACh is removed from the synaptic cleft very rapidly. This is not the case if AChE activity is not fully developed or if it is pharmacologically inhibited. Under these circumstances the decay phase of the EPC is lengthened because of repetitive binding of ACh to receptors (Katz & Miledi, 1973; Anderson & Stevens, 1973). By comparing MEPC decays in the presence and absence of AChE inhibitors, the contribution of AChE to the synaptic current decay can be measured. This is especially important in developing NMJs because very little AChE is present at newly formed synapses.

Acetylcholine Receptors

Function

The functional properties of AChR channels have been revealed in greatest detail by the recently developed patch clamp technique (Hamill *et al.*, 1981). This method takes advantage of the fact that an electrically tight seal can form between the plasma membrane of cells and the tip of a glass electrode. The seal is of such high resistance (10 to 100 GΩ) that virtually all current which flows through open channels in the patch of membrane sealed to the electrode must also flow through the electrode. A low concentration of agonist is added to the solution inside the recording electrode to activate AChRs. The patch is so small (about 1.0 μm in diameter) that it often contains only a few channels and currents flowing through single active channels can be

observed. AChR single channel currents are only a few picoamperes in amplitude, but can be clearly resolved in most cases. The advent of patch clamp technology has revolutionized the study of AChRs and other ion channels in excitable membranes.

One of the most striking characteristics of single channel currents is that, unlike macroscopic endplate currents, they rise and fall almost instantly and are square in appearance (compare Figures 4 and 13). Single channel records also reveal that an individual channel has the same current amplitude every time it opens. This property is very useful in identifying the channels as molecular entities. By examining the change in single channel current amplitude as the membrane voltage changes, the conductance of ion channels can be calculated.

The conductance of AChR channels varies among species and also depends on the developmental age of the muscle. In *Xenopus* (Brehm *et al.*, 1984 a,b; Kullberg & Kasprzak, 1985) and in rat (Sakmann & Brenner, 1978; Siegelbaum *et al.*, 1984; Vincini & Schuetze, 1985) at least two AChR channel types are present and the conductance of one class is about 50% greater than the other. Some workers have observed that the channel conductance occasionally steps to discrete subconductance levels (Colquhoun & Sakmann, 1981; Hammill & Sakmann, 1981; Auerbach & Sachs, 1983, 1984; Colquhoun & Sigworth, 1983; Sigworth, 1985; Morris & Montpetit, 1986). The mechanism involved in reaching a subconductance level is unknown.

Based on permeability measurements of many small organic cations, the pore size of the AChR channel is estimated to be about 6.5 Å (Hille, 1984). AChR channels reject anions but are not very selective among small cations. The membrane voltage at which single channel currents or EPCs reverse their direction is in the neighborhood of 0 mV, which does not correspond to the equilibrium potential of any of the physiologically important cations. Sodium, potassium, and calcium ions all permeate the pore well (Hille, 1984); but at the resting membrane potential the driving force for sodium is much greater than for potassium, so the net result of receptor activation is an inward movement of positive charge and a consequential depolarization of the membrane.

Another noticeable feature of single channel function is that the durations of the open and closed channel lifetimes are not constant but have random lengths. When open or closed durations are measured and compiled into histograms, they are found to be distributed as the sum of one or more exponential functions. The number of exponential components necessary to describe the open or closed durations is a lower estimate of the number of different open or closed states of the channels. Several authors (Colquhoun & Hawkes, 1983; Colquhoun & Sigworth, 1983; Colquhoun & Sakmann, 1985; Adams, 1987) have presented excellent discussions of the stochastic nature of AChR function.

The kinetic model most commonly used to describe AChR function is the following:

$$2A + R \leftrightarrow A + AR \leftrightarrow A_2R\ [\text{closed}] \underset{\alpha}{\overset{\beta}{\leftrightarrow}} A_2R^*[\text{open}]$$

where A = agonist
R = closed receptor
R* = open receptor
β = opening rate constant
α = closing rate constant

Channel openings are sometimes separated by very brief closures. It was initially proposed that flickering between open and closed states resulted from the loss of one ligand from the receptor (Colquhoun & Sakmann, 1981). However very brief closures are seen in the presence of high doses of ACh (Auerbach & Lingle, 1984) and in the absence of ACh or other agonists in spontaneously opening receptors (Jackson, 1984). These observations suggest that flickering may be independent of agonist receptor interaction. Long closures are thought to result when at least one agonist binding site is vacant (see Adams, 1987, for a discussion).

AChR channels also exhibit desensitization (Katz & Thesleff, 1957). When exposed to high (>5 μM) ACh concentrations, receptors enter a long lived nonconducting state (Sakmann, 1980). This is not due to blockage of the channel by the agonist, but may involve ACh binding to different sites than those involved in activation of the channel (reviewed by Raftery *et al.*, 1985). The physiological significance of desensitization is not clear, but it may contribute to neuromuscular blockade by some muscle depolarizing agents commonly used during surgery and to the lethal action of some insecticides and nerve-gases which inhibit endplate AChE.

Molecular structure

The structural details of the AChR are discussed in a number of recent publications. (Raftery *et al.*, 1986; Raftery *et al.*, 1986; Anderson, 1987; Karlin, 1987; Maelicke, 1987; Stroud, 1987). There is an emerging agreement regarding the general features of AChR structure but its topological organization with respect to the membrane is still in question (Guy, 1987; Guy & Hucho, 1987).

Most of the current knowledge of receptor structure comes from studies of AChRs from the electric organ of *Torpedo* or *Electrophorus*. The electric organ is a modified muscle which is sufficiently rich in AChRs to allow conventional biochemical techniques to be employed in probing the structure and determining the functional regions of the receptor. The electric fish receptor is a pentamer composed of 4 different subunits, α, β,

γ, and δ, with a stoichiometry of $\alpha_2\beta\gamma\delta$. All of the subunits are integral polypeptide chains which are arranged in a rosette around a central pit believed to be the opening of the ion channel. The subunit arrangement is uncertain, but the two α's are probably separated by other subunits. The general view of the subunit association is like barrel staves around a central pore which extends all the way through the protein complex.

The molecular weight of the whole receptor protein is about 280 kD and the subunits range from about 50 kD to 60 kD (Karlin *et al.*, 1987). The extracellular surface of the protein is conjugated to sugars and there is some indication that fatty acids may also be bound to two of the subunits. The dimensions (Karlin *et al.*, 1987) of the whole receptor protein are about 85 Å across on the extracellular surface and 110-140 Å in length, with 50-70 Å protruding from the plasma membranes surface and 20-30 Å into the cytoplasm.

AChR subunits have fundamental similarity in their structure and domain distribution. Each subunit is characterized by an extracellular N-terminus, a cytoplasmic C-terminus, and 5 α helical domains which are probably membrane spanning regions. One is amphipathic and the other four are hydrophobic. The original models of Finer-Moore and Stroud (1984) and Guy (1984) propose that all of the five subunits contribute the polar side of their amphipathic helices to form a water-filled pore which serves as the ion channel and that the hydrophobic helices are packed around the pore to provide stability within the membrane. These models of the ion channel are now being challenged by new evidence. Photolabeling experiments using a noncompetitive blocker indicate that the channel is lined with hydrophobic helices from each subunit, with each helix having charged amino acids only at each end (Hucho *et al.*, 1986). Questions also arise from findings that antibodies directed against various regions of the polypeptide chains bind at extracellular or intracellular locations which are different from those predicted by the original models (reviewed by Guy, 1986). At present there is no consistent interpretation of the new experimental findings, and until these conflicts are resolved the structure of the ion channel will remain unclear.

The genes for the different AChR subunits have been cloned and sequenced (Noda *et al.*, 1983 a,b,c; LaPolla & Mayne, 1984; Boulter *et al.*, 1985; Kubo *et al.*, 1985; Takai, 1985; & reviewed by Heinemann *et al.*, 1987). The four types of polypeptide chains are encoded by different genes and each of the chains is distinguishable immunologically (Lindstrom *et al.*, 1979). About 40% of their amino acid sequences are similar (Noda *et al.*, 1983) which suggests that the subunits are homologous and that the primitive AChR was a homooligomer. Gene duplication during evolution may have resulted in divergence of the subunit chains. Based on the sequences of the cloned genes, it is believed that this occurred in two steps (discussed by Anderson, 1987). Initially present was a γ-δ prototype, which gave rise to an α-β chain. Later α diverged from β and γ from δ. The

basic pentameric structure is believed to be highly conserved from fish to mammals (Raftery *et al.*, 1983), which indicates that divergence of the subunit genes was an early evolutionary event.

It is clear that the different subunits do not make identical contributions to receptor function. Only the α subunits contain agonist binding sites involved in activation of the receptor (Karlin *et al.*, 1975), and the γ subunit regulates certain gating and conductance properties during development (Mishina *et al.*, 1986). Changeaux (1984) has suggested that the selective advantage of the present form of the receptor over the homooligomer may be the ability to achieve finer allosteric regulation.

Recent work by Grenningloh *et al.* (1987) and Schofield *et al.* (1987) strongly suggests that the genes encoding the AChR subunits may belong to a super family of genes which code for the subunits of other neurotransmitter activated channels as well. These two groups have clearly demonstrated that the glycine receptor and the GABA (γ -aminobutyric acid)/benzodiazepine receptor, both of which are chloride channel proteins, are related to the nicotinic AChR. Analysis of sequences from cDNAs indicates that the subunits of these chloride channels and the subunits of AChRs have similar amino acid sequences and a remarkable similarity of domain distributions. Both the glycine and the GABA/benzodiazepine receptor subunits have hydrophobic regions in the same positions as AChR subunits and probably have the same topology with respect to the membrane. Both chloride channels also have some very specific structural features in common with the AChR including a highly conserved Cys-Cys domain in the extracellular region and a ligand binding site with closely spaced cysteines (Kao, 1984).

It is likely that the common structural features observed in the subunits of these channels reflect their functional similarities. All three groups of subunits form neurotransmitter gated ion channels, which apparently need to differ only slightly in order to recognize different agonists or to allow different ions to permeate their channels. Grenningloh *et al.* (1987) and Schofield *et al.* (1987) suggest that all of the genes coding for different neurotransmitter receptors may belong to two evolutionarily distinct gene families. One family evolved from an ancestral gene coding for receptors with an integral ion channel such as the ACh, GABA, and glycine receptor channels and the other for receptors coupled to G-proteins, such as muscarinic ACh and β-adrenergic receptors. As the relationship between the nicotinic AChR and other ligand activated channels is elucidated it will be very interesting to compare the regulation of their function and gene expression.

Acetylcholine receptor gene expression

Coordinating the expression of receptor genes is not a simple task for the muscle cell, because each subunit is encoded by a different gene (Anderson & Blobel, 1981; Noda *et al.* 1982, 1983c). In the only comparable system where control of gene expression is fairly well understood, namely the globin genes, transcriptional control has been found to be very different for the α and β subunits (Charnay, 1984). If AChR genes also have different regulation, the coordinate gene expression necessary for the biosynthesis of a complete receptor may prove to be a difficult process to elucidate.

Subunit genes are probably single copy genes (discussed by Anderson, 1987). In the chick, at least two of the subunit genes reside on the same chromosome and are separated by only about 750 base pairs (Nef *et al.*, 1984). This linkage may reflect the existence of a regulatory locus in the same region of the DNA which acts on both genes to achieve coordinate expression (Anderson, 1987). The degree of linkage between the other subunit genes is not known at present.

AChR proteins appear on the muscle surface at about the same time in development as muscle specific contractile proteins (Fischbach & Cohen, 1973; Land *et al.*, 1977) and their corresponding mRNAs (Hastings & Emerson, 1982). This temporal correlation suggests the possibility that AChR genes are coordinately regulated as part of a battery of genes during myogenesis. However, recent work suggests that α subunit mRNA may be constitutively expressed at low levels in undifferentiated BC3H-1 cells (Olson *et al.*, 1984; Merlie & Lindstrom, 1983). It is not presently known if this is true for the other chains as well. Possibly transcription of the other subunit genes limits the rate of receptor assembly and appearance on the membrane. By using the appropriate probes to the other subunit mRNAs it should be possible to determine if processes other than transcriptional control determine the rate of receptor expression.

AChR mRNA concentration is greatly increased during development, paralysis or denervation (Merlie *et al.*, 1984; Goldman *et al.*, 1985, Klarsfield & Changeux, 1985; reviewed by Salpeter, 1987b). AChR levels are increased by extracellular signals such as soluble factors from brain (Podleski *et al.*, 1978; Jessel *et al.*, 1979; Knaack & Podleski, 1985) and by specific neuropeptides (New & Mudge, 1987), but electrical or contractile activity of muscle apparently suppresses AChR synthesis (reviewed by Fambrough, 1979; Schuetze & Role, 1987). The molecular mechanisms for controlling the rate of receptor synthesis and insertion are not understood. It is not clear how mRNA levels are regulated or how they are related to the rate of appearance of mature receptors on the membrane.

The mechanisms controlling the extensive postranslational modifications, coordinated intracellular transport of subunits and assembly to form mature receptors are mostly speculative; however, the chronology of events in receptor synthesis is reasonably well understood (reviewed by Merlie & Smith, 1986; Anderson, 1987). In summary, the polypeptide chains are synthesized on rough endoplasmic reticulum and contain a signal peptide which is cleaved prior to maturation of the chain. The α chain (and possibly the others) requires about 15 minutes after translation to achieve a mature conformation which is recognized by a monoclonal antibody to the native receptor or by α-BTX. The maturation of the α subunit apparently occurs independently of any association with the other receptor subunits. It is not known in which intracellular compartment maturation of the chain occurs. Interestingly, less than one third of α subunits reach maturity *in vivo*, and the rest are rapidly degraded (Merlie *et al.*, 1982). The control of chain maturation might determine the abundance of subunits capable of assembly (Merlie, 1984). Olson *et al.* (1983) observed that a down regulation in assembly of complete ACh receptors in BC3H-1 cells was accompanied by a decrease in α chain maturation, whereas synthesis of the α chain was unabated. However, this result was obtained from immortalized cells in culture, and Anderson (1987) points out that changing the proportion of chains achieving maturation has not been shown to be important in regulation of AChR synthesis *in vivo*.

Subunits are known to be modified postranslationally by N-glycosylation, O-glycosylation, fatty acylation, and disulfide bond formation. (Anderson, 1987). Most of these modifications are thought to occur in the Golgi. The only functional significance that has been demonstrated for any of these modifications is disulfide bond formation near the putative ACh binding site and N-glycosylation of the α subunits. ACh is not effective in opening the channel if an S-S bond near the ACh binding site is reduced (Kao *et al.*, 1984) or if one of the cysteines is replaced by a serine (Mishina, 1985). Conti-Tronconi *et al.* (1984) have shown that differences in the extent of N-glycosylation are correlated with differences in the affinity of the two α subunits for agonist.

After the α chain is mature, there is a lag of about one hour until the first association between subunits can be detected (Merlie & Lindstrom, 1983). It is likely that the different subunits assume their quaternary relationship in the Golgi. During movement of the subunits from the cis to the trans Golgi, they become more concentrated and less membrane area is available to them. These conditions could favor the process of receptor assembly, as has been demonstrated for other membrane proteins (Quinn *et al.*, 1984). There has been no demonstration that other proteins participate in the assembly of subunits, which argues that assembly may occur autonomously. Subunits which are not assembled into receptors are probably directed to lysosomes from the trans Golgi and are degraded (Merlie *et al.*, 1982).

From the Golgi, receptors are packaged in coated vesicles to be transported to the plasma membrane (Bursztajn & Fischbach, 1984). It has been reported that AChRs and AChE are co-transported in the same vesicles in embryonic cells (Porter-Jordan *et al.*, 1986). The plasma membrane site where the receptors are initially deposited is uncertain. Receptors in embryonic muscle can be found in abundance over the entire surface of the muscle, whereas they are concentrated almost exclusively at the synapse in adult muscle (Salpeter, 1987a). Some possible mechanisms for this segregation of AChRs during development are discussed below.

Acetylcholinesterase

Two distinct classes of AChE, globular and asymmetric, have been recognized (reviewed by Rotundo, 1987; Brimijoin, 1983). Both forms exhibit polymorphism. The globular forms are composed of similar sized subunits and are found as monomers, dimers and tetramers. The asymmetric forms have a collagen-like tail attached via a disulfide bond to one, two or three tetramers. The asymmetric form is found at the NMJ in high density where it is attached by its collagen-like tail to the synaptic basal lamina (Bon and Massoulie, 1978; Torres & Inestrosa, 1983). The asymmetric form is virtually absent from synapses in the CNS, which might be expected due to the lack of synaptic basal lamina in central synapses. Globular forms of the enzyme may also be present at the NMJ, but in electric fish it is reported to be exclusively associated with the presynaptic plasma membrane (Li & Bon, 1983). The globular forms vary from hydrophobic to completely water soluble. Increased hydrophobicity is produced by conjugation of a glycophospholipid to the C-terminal end of the polypeptide chain.

Recent analysis of the amino acid sequences deduced from cDNAs encoding the catalytic subunits of the asymmetric form of AChE has revealed important features of the enzyme (reviewed by Taylor *et al.*, 1987). The hydrophobicity profile indicates that the catalytic subunits are much more typical of secreted glycoproteins than integral membrane proteins such as the ACh receptor. The AChE molecule shows no homology with other catabolic enzymes such as trypsin, nor to the α subunit of the AChR despite a similar specificity in binding ACh. Although the functional relationship is not evident, AChE has a high degree of sequence similarity to the C-terminal domain of thyroglobulin.

An interesting similarity to AChR synthesis has recently been discovered. Like the α subunit of the AChR, the newly synthesized AChE chains are not mature (based on their catalytic activity) for about one half hour after their synthesis. Rotundo (1987) has suggested that this lag in maturation is probably required for conformational folding of the AChE molecule into its active form. The time course of AChE biosynthesis is an important consideration in understanding the possible coordinate regulation of AChE and other synapse-specific proteins during development.

Development of the Neuromuscular Junction

Chronology

In recent years some general features about the timetable of NMJ development have emerged. Synapse formation begins early in embryonic development; the pre- and postsynaptic cells engage in neurotransmission upon first contact, and formation of the fully mature synapse requires a relatively long time (reviewed by Fambrough, 1979; Fischbach *et al.*, 1979; Cohen, 1980; Dennis, 1981; Bennett, 1983; McArdle, 1983; Salpeter & Loring, 1985; Salpeter, 1987; Purves & Lichtman, 1985). These points are exemplified by the development of the myotomal neuromuscular junction in *Xenopus*. In developing myotomal muscle, motor neurons contact the muscle (Kullberg *et al.*, 1977; Chow & Cohen, 1983) and initiate synaptic transmission within one day of fertilization (Kullberg *et al.*, 1977). Within about four hours after initial contact the nerve begins to induce muscle movement (Blackshaw & Warner, 1976a; Kullberg *et al.*, 1977). It is likely that neurotransmission begins almost immediately after the growth cone reaches the muscle (Kullberg *et al.*, 1977). Although the structural specializations which characterize the NMJ are not present at initial contacts (Kullberg *et al.*, 1977; Salpeter, 1987) some of the components involved in synaptic transmission are already in place. It has been shown that ACh is released by growth cones in cultured *Xenopus* neurons (Young & Poo, 1983; also see Hume *et al.*, 1983) This indicates that growth cones already possess the ability to release transmitter even before contacting the target cell. The postsynaptic membrane is also prepared to respond to transmitter release by the time the growth cone arrives. In developing myotomal muscle, AChRs are present on the muscle's surface prior to the arrival of the nerve (Kullberg *et al.*, 1977; Chow & Cohen, 1983; see Results) and synaptic currents can be recorded at about the time nerve-muscle contact first occurs (Kullberg *et al.*, 1977).

In *Xenopus*, synaptic currents do not acquire their mature wave-form for about one week after neuromuscular transmission begins (Kullberg, 1980; see Results), which is a relatively long time for the rapidly developing *Xenopus* larvae. In other animals as well, NMJ maturation requires a protracted period of time, sometimes as long as several weeks (Purves & Lichtman, 1985; Salpeter, 1987).

Morphological differentiation

When the growth cone and the muscle cell make initial contact, electron microscopic examination reveals none of the specializations typical of the mature synapse in either the pre- or postsynaptic elements (Kullberg *et al.*, 1977). The presumptive nerve terminal resembles a growth cone, both in gross morphology and ultrastructure. As the

growth cone differentiates into a presynaptic terminal, active zones develop and there is an associated increase in mitochondria, microfilaments, and synaptic vesicles (Kullberg *et al.*, 1977; Salpeter, 1987b).

The space between the terminal and the muscle cell is initially irregular and contains little basal lamina. As development proceeds, the cleft acquires a more constant width and the synaptic basal lamina becomes distinct. The synaptic basal lamina is antigenically distinct and there is strong evidence that it can direct the differentiation of terminals and postsynaptic membrane in regenerating muscle (reviewed by Burden, 1987). The role of the basal lamina in developing synapses is not yet known, but it has been found that components of the basal lamina are added, lost or modified differentially in synaptic and extrasynaptic regions during development (Chiu & Sanes, 1984). Since the basal lamina is absent early in synaptogenesis, its role may not be important in determining initial nerve-muscle contact but rather in helping to direct or support later differentiation.

One of the major basal lamina components in the mature NMJ is AChE (Burden, 1987; Rotundo, 1987). There is little AChE localized at the newly formed synapse, but it progressively accumulates in the cleft. In myotomal muscle, AChE localization is very rapid after synapse formation. It reaches its full physiological complement within about one day after NMJ formation (Kullberg, 1981; see Results). In the chick (Kato *et al.*, 1980; Toutant *et al.*, 1983) and rat (Koenig & Rieger, 1981) it has been shown that AChE activity exists in presumptive muscle cells before innervation, but it is only in the globular form. During this early period, AChE activity can be detected histochemically over the entire muscle membrane (Wake, 1976). After synaptogenesis, extrajunctional AChE activity diminishes and the asymmetric form of the enzyme becomes progressively more abundant and selectively accumulates at the synapse.

The postsynaptic membrane is relatively smooth at first but characteristic thickenings soon develop at the tops of the junctional folds. These thickenings may result from a high concentration of subsynaptic proteins in the sarcoplasm and from AChRs anchored in the postsynaptic membrane. The aggregation of subsynaptic proteins may be involved in maintenance of the postsynaptic architecture (reviewed by Froehner, 1986). One of the major proteins associated with this function is the so called "43 kD protein" which colocalizes with AChR clusters (reviewed by Burden, 1987; & by Schuetze & Role, 1987). Its developmental appearance coincides with the first clustering of AChRs in *Xenopus* muscle (Burden, 1985) and can also be found at AChR clusters which form in aneural cultures (Burden, 1985; Peng & Froehner, 1985). This protein may anchor AChRs to the cytoskeleton and restrict their mobility, a condition essential to the maintenance of receptor localization at the synapse.

The distribution and concentration of postsynaptic AChRs is obviously important to the function of the synapse. The general features of AChR distribution in different species have been the subject of several reviews (Fambrough, 1979; Salpeter & Loring, 1985; Schuetze & Role, 1987; Salpeter, 1987b). Of particular relevance to the present study is a recent investigation by Chow & Cohen (1983) of AChR distribution in developing *Xenopus* myotomal muscle. They visualized AChRs by binding fluorescent-labeled α -BTX to them. By measuring the uptake of ^{125}I-α-BTX they found that AChRs exist on the muscle membrane prior to innervation. However, at this time fluorescent-labeled toxin did not localize in patches, indicating that the receptors were uniformly distributed on the muscle membrane. Soon after the nerve contacted the muscle, AChR density progressively increased and discrete patches of receptors appeared at the ends of the muscle cells where synapses are known to be located. The onset of receptor localization at the ends of the cells was very rapid. They observed discrete patches of receptors about three hours after innervation. The total density of receptor sites started to diminish at about the time of hatching and continued to decline during maturation of the endplate. This decline was probably due mainly to the loss of extrajunctional receptors, because the density of junctional receptors remains relatively constant (Matthews-Bellinger & Salpeter, 1983; H. Kasprzak, personal communication).

It was traditionally thought that "junctional" and "extrajunctional" receptors differed in function as well as location on the muscle membrane. However, recent evidence argues that receptor properties are not dependent on location (Kullberg & Kasprzak, 1985; Brehm & Kullberg, 1987; see Results) and that the genes encoding for extrajunctional and junctional receptors are not different (reviewed by Merlie & Sanes, 1986; Merlie & Smith, 1986; Anderson, 1987). There are some distinct differences in the regulation of their distribution and number, however (reviewed by Salpeter & Loring, 1985; Salpeter, 1987b; Schuetze & Role, 1987). Extrajunctional receptors are much more mobile in the plane of the membrane than those at the junction. Extrajunctional receptors can diffuse relatively freely in the lipid bilayer (reviewed by Poo, 1985), while receptors at junctions are resistant to dispersal. The immobility of junctional AChRs appears to be a developmentally acquired characteristic. If innervation is prevented (Braithwaite & Harris, 1979; Creazzo & Sohal, 1983) or denervation occurs very early (Harris, 1981), clusters of AChRs disperse. Localization of AChRs at the junction is thought to occur by trapping mobile extrajunctional receptors (reviewed by Fraser & Poo, 1982) and also by selective insertion of receptors at the junction. The former idea is supported by the observation that fluorescent-labeled extrajunctional receptors become localized at the synapse (Anderson *et al.*, 1977), while the latter idea arises from the finding that AChR mRNA and newly formed AChR proteins are localized in intracellular regions of the muscle very close to the synapse (Merlie & Sanes, 1985; Pestronk, 1985; Merlie & Sanes, 1986; Merlie *et al.*, 1987). A number of researchers are now investigating different factors which can induce AChR clustering or AChR-AChE colocalization *in*

vitro (Fallon *et al.*, 1985; Wallace *et al.*, 1985; Usdin *et al.*, 1986; Wallace *et al.*, 1986; reviewed by Burden, 1987). It is not yet known how important these factors may be in organizing the developing synapse *in vivo*.

At about the same time that junctional receptors become resistant to dispersal their metabolic stability also increases. Embryonic junctional and nonjunctional receptors in chick and mammals have a degradation half life of about one day or less, but for adult junctional receptors it is over one week (reviewed by Fambrough, 1979; Salpeter & Loring, 1985; Salpeter, 1987b). In *Xenopus* the turnover rate of embryonic receptors is two to three fold slower than in chick or mammals (Brehm *et al.*, 1983). Data on turnover at mature junctions in amphibians is not yet available.

Innervation causes a slowing of the degradation rate of receptors at the junction (Burden, 1977; Reiness & Weinberg, 1981; Steinbach, 1981) but has no effect on the degradation rate of extrajunctional receptors (Steinbach, 1981). The dramatic decline in density of extrajunctional receptors during development of muscle is presumably due to a decrease in the rate of receptor synthesis. The cumulative data suggest that receptor synthesis is regulated by innervation and electrical or contractile activity of the muscle (Merlie & Sanes, 1986). When the muscle is denervated or paralyzed, receptor synthesis increases many fold and extrajunctional AChR density increases accordingly (Salpeter, 1987b). It has recently become evident that other synapse-specific proteins are coordinately expressed with AChRs during synaptogenesis, suggesting mutual regulation; and it also appears that induction of synaptic proteins may differ in mature and developing muscle (Merlie & Sanes, 1986; Merlie *et al.*, 1987).

AChE may be mutually regulated with ACh receptors during synaptogenesis (Merlie & Sanes, 1988). There are some interesting parallels in development of the two molecules (reviewed by Rotundo, 1987). In the rat and chick only the globular form of the enzyme is found in the embryonic muscle prior to synaptic contact. With innervation, the asymmetric form gradually becomes more abundant, and like AChRs in embryonic muscle, it is uniformly distributed over the muscle's surface. With continued maturation, the asymmetric form becomes almost exclusively localized at the synapse. The similarity in distribution of AChE and ACh receptors in developing muscle is not surprising since it was recently shown that the two molecules are cotransported in coated vesicles to the membrane in developing muscle cells (Porter-Jordan *et al.*, 1986).

Development of postsynaptic function

Synaptic currents from newly formed junctions decay as much as eight times more slowly than those from mature muscle (Diamond & Miledi, 1962; Bennett & Pettigrew, 1974; Kullberg *et al.*, 1977; Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980;

Kullberg *et al.*, 1980; Michler & Sakmann, 1980; Vincini & Schuetze, 1985; Schuetze & Vincini, 1986; see Results). Synaptic currents also become briefer after reinnervation of denervated adult muscle (Miledi, 1960; Bennett *et al.*, 1973; Brenner & Sakmann, 1978) and after innervation of muscle cells cocultured with neurons (Robbins & Yonezawa, 1971; Cohen, 1972).

The two features of endplate development which contribute most to the increase in the rate of MEPC decay are the accumulation of AChE at the junction and a developmental decrease in open time of AChR channels. When AChE is inhibited at mature junctions, synaptic currents are prolonged because individual molecules of ACh can bind repeatedly to receptors before diffusing away (Katz & Miledi, 1973). AChE activity is very low or absent at newly formed junctions (Bennett & Pettigrew, 1974; Bevan & Steinbach, 1977; Kullberg *et al.*, 1980; see Results), which suggests that the slow decay of synaptic currents at developing synapses may also be due to repeated binding of ACh to receptors.

Analysis of synaptic current decay, ACh induced noise, and single channel records from extrajunctional membrane have shown that two receptor classes exist on developing mammalian and frog muscle (Sakmann & Brenner, 1978; Kullberg *et al.*, 1981; Brenner & Sakmann, 1983; Brehm *et al.*, 1984a,b; Kullberg & Kasprzak, 1985; Vincini & Schuetze, 1985, Schuetze & Vincini, 1986; Brehm *et al.*, 1987; see Results). In general the first AChRs to appear on the embryonic muscle have a low single channel conductance (40 pS) and long mean channel open time (~3 ms). As development proceeds a second class of channels with about 50% higher single channel conductance and three to five fold shorter mean channel open time progressively replaces the smaller channel. The high conductance channel is dominant in mature twitch muscle (reviewed by Schuetze & Role, 1987). The cumulative data suggest that the shortening in synaptic current decay is due not only to the development of AChE but to the increased gating speed of junctional receptors. However, this has not been directly confirmed by recording single channel activity at the developing neuromuscular junction.

Specific Background to the Present Research

In the previous pages, a general introduction to neuromuscular transmission has been presented. Next, the specific background to the present research will be discussed.

Development of acetylcholine receptors

In general, changes in the single channel properties of ACh receptors involve a developmental increase in channel conductance and a decrease in the channel open time. Changes in the gating properties of AChRs in *Xenopus* were first revealed by analysis of

ACh noise recorded from muscle (Kullberg, *et al.*, 1981; Kullberg & Kasprzak, 1985). These experiments gave information on the chronology of change during normal muscle development *in vivo*, but provided no information about the conductance properties of the channels. Later experiments, using single channel recordings, characterized developmental changes in the conductance classes of AChR channels and their gating properties in aneural muscle cells grown *in vitro* (Brehm *et al.*, 1984a; Leonard *et al.*, 1984). These studies gave important information about the development of different AChR conductance classes and their associated gating properties. However, it is not known how closely AChR development in culture resembles that which occurs in normally developing, innervated muscle *in vivo*. Clearly needed for our understanding of AChR development is a detailed description of the single channel properties of AChRs and their changes throughout the normal *in vivo* development of muscle.

The aim of this work was to describe the single channel properties of AChRs in *Xenopus* myotomal muscle during the normal development *in vivo*. Myotomal muscle was chosen for this study because of the extensive data available on synaptic currents and AChR development in this muscle, both *in vivo* and *in vitro* (Kullberg *et al.*, 1977; Kullberg *et al.*, 1980; Kullberg *et al.*, 1981; Brehm *et al.*, 1982; Brehm *et al.*, 1984a,b; Leonard *et al.*, 1984; Kullberg & Kasprzak, 1985). The gating and conductance properties of AChRs on nonjunctional myotomal muscle membrane were studied throughout the full range of muscle development. The age span of this study extended from the time the receptors first appeared in embryos, less than one day old, until the muscle was lost at metamorphosis. This study is the first to describe in detail the single channel properties of AChRs during normal muscle development *in vivo*.. In the Discussion, AChR development in the intact animal is compared with results obtained by others in cell culture, and the temporal relationships are examined between developmental changes in AChR function and motor innervation, synaptic function, muscle movement and other features of muscle development. The possible significance of expressing different channel types during development is discussed.

Role of activity in the development of endplate currents in myotomal muscle

Studies of the developing myotomal synapse in embryos of *Xenopus laevis* have revealed an orderly series of events beginning with the first nerve-muscle contact and continuing through maturity of the synapse. As mentioned above, these events include the aggregation of acetylcholine receptors at the site of nerve-muscle contact (Anderson & Cohen, 1977; Anderson, *et al.*, 1977), the deposition of junctional acetylcholinesterase (Kullberg, *et al.*, 1980) and a reduction in open time of ACh receptor channels (Kullberg *et al.*, 1981; Kullberg & Kasprzak, 1985). Synaptic currents change in duration on a predictable schedule reflecting increased AChE activity and reduced gating time of

ACh receptor channels. These events are similar to those reported in developing rat and chick skeletal muscle (reviewed by Dennis, 1981; Bennett, 1983) with the exception that channel gating time does not change in chick (Schuetze, 1980).

The mechanisms controlling these developmental events are a subject of much interest. One possible controlling factor which has been investigated is the electrical or contractile activity of muscle. It appears that the aggregation of receptors at the site of neural contact proceeds normally in the absence of muscle activity (Cohen, 1972; Anderson & Cohen, 1977; Anderson *et al.*, 1977; Obata, 1977; Rubin *et al.* 1980), but there is evidence that the deposition of the endplate-specific form of AChE, at least in rat and chick, is dependent on muscle activity (Rubin *et al.*, 1980; Rieger *et al.*, 1980; Betz *et al.*, 1980; Brockman *et al.*, 1983). In developing amphibia, the influence of muscle activity on AChE levels may be less than in rat or chick skeletal muscle. This is indicated by a recent study of developing *Xenopus* myotomal muscle which revealed that chronic exposure to a paralyzing anesthetic (tricaine) did not prevent the appearance of AChE (Cohen *et al.*, 1984). There is relatively little knowledge about the role of muscle activity in the development of ACh receptor channel function. The only evidence so far comes from tissue cultured *Xenopus* muscle, which indicates that the development of fast gating kinetics does not depend on nerve-induced movement (Brehm *et al.*, 1982; Brehm *et al.*, 1984).

To investigate the effects of chronic immobilization on the development of synaptic function, endplate currents were studied in embryos and tadpoles of *Xenopus laevis* which were chronically immobilized by TTX. This toxin was used because of its specific and well-characterized action on voltage-gated sodium channels (Kao, 1966; Catterall, 1980). The aim was to determine if immobilization resulted in any change in rise times or decay constants of MEPCs. These parameters are useful measures of the normal development of synaptic function because they reflect not only AChE activity and ACh receptor channel open time but also all other kinetic processes underlying synaptic transmission such as the release and diffusion of transmitter, transmitter-receptor binding, and the transition rate from closed to open state of channels. Major disturbances in any of these processes could alter the MEPC time course. As described in the Results, it was found that the time course of synaptic currents was not affected by chronic immobilization, indicating that the ACh receptor channel open time, AChE activity and probably the other kinetic processes underlying MEPCs developed normally in the absence of muscle activity.

Comparative development of endplate currents in different muscles

In adult amphibian skeletal muscle, the gating kinetics of acetylcholine receptors differ according to muscle fiber type (Miledi & Uchitel, 1981; Fedorov *et al.*, 1982). AChR

channel open times are 3-fold longer in slow muscle fibers than in fast twitch fibers, and this difference gives rise to endplate currents of comparatively long duration in slow muscle. Intermediate channel open times and endplate currents of corresponding duration are present in some twitch fibers with multiple innervation, such as in the submaxillaris muscle (Miledi & Uchitel, 1981) and the singly innervated fibers in tonic bundles of ileofibularis and cruralis (Fedorov *et al.*, 1982). Levels of acetylcholinesterase activity are also reported to differ according to fiber type (Lannergren & Smith, 1966), with comparatively low levels being present in slow muscle.

The existence of such differences at adult endplates implies that variations in the development of function should also be found at immature endplates of different muscles. Much information has been gained about the development of endplate function in amphibian myotomal muscle (reviewed by Cohen, 1980; & by Dennis, 1981), but little is known about other developing amphibian muscles. An examination of other muscles should reveal at least as much variability in development of neurotransmission as there is in the function of adult endplates. In amphibia it is of particular interest to examine synapse development in muscles which are present in both larval and adult animals. The functions of such muscles may change during metamorphosis and it is of interest to determine if there is a corresponding change in synaptic function.

To examine the correspondence between synapse development and muscle function, we did a comparative study on the development of endplate currents in two muscles of *Xenopus laevis* which are functionally diverse and which persist through metamorphosis: the superior oblique and interhyoideus. The former is an extraocular muscle which is responsible for rapid, saccadic movements of the tadpole eye. The interhyoideus is a broad, flat muscle of the hyoid arch, which elevates the floor of the mouth (Sedra & Milad, 1957; Gradwell, 1968). Its rhythmic activity in the tadpole contributes to irrigation of the buccal cavity. Both muscles become active at about the same time in *Xenopus* tadpoles, allowing a convenient comparison of their developmental schedules. As a means of studying the development of AChR gating kinetics and AChE activity, we have described the time courses of endplate currents and their response to anticholinesterase treatment. It was found that each muscle has a distinctive program of endplate current development which contrasts with that of the other muscle.

Some of the results presented in this thesis have already been published in abstracts (Kullberg *et al.*, 1984; Owens & Kullberg, 1985; Owens & Kullberg, 1986; Owens *et al.*, 1987) and papers (Kullberg *et al.*, 1985; Kullberg & Owens, 1986; Kullberg *et al.*, 1986; Brehm *et al.*, 1987), which I co-authored.

METHODS

Animals

All experiments were performed on embryonic, larval or adult *Xenopus laevis* (South African clawed frog) ranging in age from 21 h to 2 years. This species is easily reared and mated in the laboratory. The embryos develop quickly and are relatively tolerant of experimental manipulations. They withstand premature hatching, heal quickly and are relatively resistant to infection. For these reasons *Xenopus* has been the preparation of choice for many developmental studies. This animal was especially suitable for our studies because it was possible to directly observe the embryos throughout development. Furthermore, some neurotoxins, such as TTX, when added to the bath will enter the animal and exert specific effects on neural function. This permits selective interference with nervous system function without invasive techniques. Only healthy, precisely staged animals were used in this for study. Staging of embryos and tadpoles was done according to the criteria of Nieuwkoop and Faber (1967). Fig. 1 illustrates the appearance of developing tadpoles at selected stages.

Embryos were produced by inducing mating of adult pairs with subcutaneous injections of human chorionic gonadotropin (500 IU for females; up to 300 IU for males). Some of the older tadpoles (stages 58-65) and adults used for experiments were obtained commercially (Nasco U.S.A.). Mating pairs of adult animals were kept in 5 gallon aquaria containing dechlorinated tap water. Fertilized eggs were placed in dechlorinated tap water with no salts added. Embryos and younger tadpoles (less than stage 46) were kept in 35 or 50 mm petri dishes at about a dozen per dish. Their rate of development was significantly influenced by temperature; therefore, embryos or young tadpoles used for experiments were incubated at 22-23 °C for the first 4-5 days. Thereafter the animals were maintained at room temperature which generally was from 20-24 °C. They were kept in 4 gallon aquaria at a density of 1-2 hundred per aquarium. Aquaria housing tadpoles from about 2 weeks of age through metamorphosis were equipped with biological sponge filters or undergravel filters. Under these conditions the animals developed well and at the approximate rate expected from Nieuwkoop and Faber (1967). Tadpoles and adults were fed frog brittle (Nasco frog food or Carolina tadpole food). Adults were fed twice weekly.

Description of Muscles

The development of postsynaptic function was studied in three muscles with distinctly different functional properties and roles. These muscles are the superior oblique (an extraocular muscle), the interhyoideus (a jaw muscle) and myotomal muscle (tail muscle). Each of these muscles is readily accessible when the skin is removed. Both superior

oblique and interhyoideus muscles persist through metamorphosis; however, there is evidence which suggests that larval muscle fibers are replaced by adult fibers during metamorphosis (Alley & Cameron, 1983). Motorneurons innervating the larval muscles probably persist through metamorphosis and innervate the adult muscles (Alley & Barnes, 1983).

Myotomal muscle

The myotomal muscle is the first muscle to develop and can be discerned within one day (about stage 17) after fertilization. The cells of the myotomal muscle are short (200 μm) twitch fibers arranged in myotomes along the length of the tail. The appearance of the developing myotomal muscle is illustrated in Fig. 1. Synapses are located on each end of the cell near the myocommata (Chow, 1980; Chow & Cohen, 1983; Kullberg *et al.*, 1977). Myotomal cells remain uninucleate until the onset of metamorphosis (stage 48-50), then as metamorphosis proceeds they become multinucleate, probably from fusion with satellite cells (Muntz, 1975). Myotomal muscle is resorbed completely by the end of metamorphosis and unlike the other two muscles has no adult form in the frog.

Superior oblique

The superior oblique becomes distinct at about 2 1/2 days of age (stage 39-40). This muscle is homologous to its counterpart in other vertebrates. It originates rostrally and medially to the eye and inserts on the dorsal sclera (Fig. 2). The position of the muscle does not change during development. It is innervated by the trochlear nerve and is this nerve's sole target. The trochlear nerve can be clearly visualized under low power (40X) through the transparent skin in older tadpoles.

Interhyoideus

The paired interhyoideus muscles originate at a midline tendinous junction and insert laterally on the ceratohyalia (Sedra and Milad, 1957). They are the dominant muscles on the floor of the mouth in the premetamorphic tadpole. During metamorphosis, they move caudally and acquire new insertions on the palatoquadrate cartilages. The intermandibularis muscle, while small in the tadpole, is much larger than the interhyoideus in the adult and dominates the floor of the frog's mouth. The shape and position of these muscles in the tadpole and the frog is illustrated in Fig. 3. The interhyoideus muscles are innervated by branches of the hyomandibularis nerves (Nieuwkoop & Faber, 1967).

Preparation of Muscle for Recording

Prior to dissection of muscles for recordings, embryos and tadpoles were placed in a solution containing 1-2 μg/ml TTX, which both immobilized and anesthetized them. In addition, in all animals older than about 36 h, the brain was either removed or pithed before dissection. Adults used for experiments were anesthetized by cooling to about 4 °C before pithing.

The muscles of interest were exposed by removing the overlying epithelium or skin. In older animals it was necessary to remove some overlying connective tissue. In tadpoles, this was done under a dissecting microscope with fine tipped forceps (Dumont No. 55) and minutia pins attached to Pasteur pipettes with wax.

No enzyme treatment of the muscles was necessary in any of the experiments except in preparation for patch clamp recording from myotomal muscle older than stage 41. High quality single channel records were readily obtained from younger myotomal muscle by removing the skin and simply exposing the muscle. This was advantageous because most of the interesting developments in AChR function had already occurred before the time that enzyme treatment was necessary. In order to remove adherent connective tissue and enhance the cleanliness of the muscle surface, older myotomal muscle was bathed in Ringer solution plus 1 mg/ml collagenase (Cooper Cl.S) for 5-30 minutes depending on age. Control experiments indicate this treatment did not affect ACh receptor function. Longer exposure to the enzyme did not improve the quality of high resistance seal formation, and very long (> 1 h) enzyme treatment tended to diminish the frequency of obtaining single channel records. Prior to recording, the collagenase solution was exchanged 2-3 times with Ringer solution.

At the time of recording the preparation was pinned to a Sylgard (Dow Corning) coated dish. Small clips fabricated from minutia pins were helpful in securing the preparation firmly to the dish. To improve visualization, a small 3 sided chamber made from Sylgard was placed around the preparation and a cover slip rested on top. This prevented impairment of optics by the meniscus around the recording electrode. The open side of the chamber allowed access for the recording electrode. The muscle was viewed under an upright compound microscope (Leitz Dialux) equipped with Hoffman interference optics at 200 X magnification. The microscope was mounted on an air-supported vibration isolation table (Barry Servabench). In some experiments with older animals, a stereo dissection microscope (Olympus) was used. Recording electrodes were mounted on Leitz micromanipulators. A separate light source (Intralux) with fiber optic light guides was occasionally used for incident illumination.

All experiments were done at room temperature (20-22 °C). Ambient temperature was monitored with a glass thermometer placed near the recording chamber.

Solutions

During dissection and recording of focal extracellular MEPCs the animal was placed in Ringer solution containing 110 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 8 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) buffer (pH 7.4), and TTX 1-2 μg/ml. For patch clamp recordings the bath solution was similar except that 120 mM NaCl and 1 mM KCl were used. Extracellular recording electrodes were filled with the same salt solutions as contained in the bath but without TTX. For single channel recordings, 500 nM acetylcholine chloride (Sigma) was added to the recording electrode. This concentration of ACh elicited sufficient AChR channel activity for convenient analysis without an excess of overlapping events.

Recording and Analysis of Focal Miniature Endplate Currents

Recordings of MEPCs were done with extracellular borosilicate glass (WPI) electrodes having internal tip diameters from 3 to 35 μm and resistances of 2 MΩ or less. Electrode tips were often fire polished in order to minimize damage to the surface of the muscle. Larger tipped electrodes were found to give better recordings from the interhyoideus because they tended to rest on the surface of the muscle, rather than breaking through the tissue. Focal MEPCs were recorded by placing the electrode tip in close proximity to a synapse. Although we use the term 'miniature endplate current' (MEPC) to describe the synaptic events, the recorded signal was an extracellular voltage which was proportional to the synaptic current. The time course of the MEPC parallels the change in postjunctional membrane conductance produced by ACh and is not affected by membrane time constant or the distance between the electrode tip and the synapse (del Castillo and Katz, 1956; Katz and Miledi, 1973). The extracellular MEPCs were amplified by a low noise differential amplifier (WPI DAM-5), band pass filtered at 3-5 kHz (Butterworth filter, 24 dB/octave roll off) and recorded on FM tape (Racal Store 4DS) at 7.5 ips.

Analysis of MEPCs was done off line by computer (DEC PDP 11/34) on records which were digitized at sample intervals of 50 to 300 μs. Up to 85 MEPCs were analyzed from each recording site, with typical sample sizes being about thirty. The mean rise time and decay constant(s) of each MEPC were measured at each recording site. The rise time was defined as the time interval between the onset of the synaptic current and the peak amplitude. The time of onset was defined as the first point to fall within twice the standard deviation of the baseline noise (which typically was about 20 μV) when the MEPC was searched backward from the peak. The decay constant was defined as the time interval required for the amplitude of the MEPC to decline e-fold. The decay phases were measured by the best fitting single or double exponential curves. Exponential curves were fitted by linear regression to the semilog transform of the

MEPC decay phase, between 10% and 90% of the peak amplitude. In the case of the double exponential MEPCs we first averaged a sample of MEPCs (up to 20) to obtain an improved signal to noise ratio. We fitted a single exponential curve to the slow component by least squares, setting the start point of the fit by eye. The fitted slow exponential was then subtracted from the averaged MEPC and the remaining fast component was fitted by least squares. All data are expressed as the mean ± s.d. Sample sizes refer to the number of recording sites. Data obtained from different animals at the same developmental stage were pooled.

Recording and Analysis of Single Channel Currents

Standard single channel recording techniques were used (Sakmann & Neher, 1985). All records were taken from nonjunctional membrane in the cell attached mode. Recordings were made with borosilicate glass (WPI) electrodes pulled to a tip diameter of about 1 μ m (resistance ~10 MΩ). The tips were bent using a microforge so that they would approach the cells vertically. Electrode tips were occasionally fire polished; however, for most experiments high resistance seals >10 GΩ formed readily without fire polishing. We found signal to noise ratios acceptable without coating the electrode with noise reducing, nonconductive materials.

Single channel currents were recorded with a List EPC/7 patch clamp, Bessel filtered at 3 kHz and recorded on FM tape. Records used for analysis were typically taken at 3-4 applied pipette voltages ranging from 0 to 120 mV. A square wave command voltage from an external stimulator (WPI Digi-Pulser Series 1800) was applied via the EPC/7 to the recording pipette in order to monitor resistance during seal formation. During entry into the bath and while approaching the tissue, positive pressure (1-4 cm water) was applied to the electrode to keep the tip clean. Seals formed when suction (0-25 cm water) was applied to the pipette. A U-shaped, water filled, glass manometer was used to measure pressure to the recording pipette. The applied pressure to the pipette was switched between atmospheric, negative, or positive with a three-way valve placed in-line.

There are several reports (Brehm *et al.*, 1984a,b; reviewed by Kullberg, 1987) that suction applied to the patch can elicit channel openings in muscle membrane. When suction was applied, we also observed bursts of channel openings in some patches, including those with AChRs blocked by 0.1 g/ml αBTX (see Results). The suction-activated channels in myotomal muscle have current amplitudes which are similar to AChR channels. To avoid confusing the two kinds of channels we routinely applied suction to the patch during the recording, and if bursts of channel activity were induced the recording was not used.

Analysis was done off-line with an INDEC 11/73 Laboratory Display System. Sections of the tape-recorded single channel data were digitized at 10 kHz and stored on disk. The amplitudes, open durations, and closed durations of the single channel records were measured automatically, followed by a manual review to check the accuracy of the measurements. The threshold for detection was set at 1/2 the channel current amplitude. Multiple events were excluded from the analysis. The briefest open and closed times accepted for analysis were 300 μs and 200 μs, respectively. Histograms of open and closed durations and current amplitudes were compiled from the data. The amplitude histograms generally had 1 to 4 well-resolved peaks, which were used to define the limits for the different amplitude classes of channel openings. After defining these limits, open duration histograms at each recording site were compiled for each amplitude class of channel openings. All open duration histograms were fitted by single exponential functions, by the method of maximum likelihood (Colquhoun & Sigworth, 1985). The goodness of fit was evaluated by eye, and those histograms which deviated noticeably from a single exponential distribution were fitted by double exponentials using a recursive maximum likelihood routine. We use the terms 'mean open time' to refer to the time constants obtained from these histograms. Unless stated otherwise, all data are expressed as mean ± standard deviation and the n values all refer to the number of recording sites.

Intracellular Recording

Resting potentials were measured with an electrometer (WPI M707) by impaling muscle cells with fine-tipped microelectrodes. The intracellular electrodes were filled with 3 M KCl and their resistances ranged from 20 to 40 MΩ. Intracellular voltages were monitored with a storage oscilloscope (Tetronix) and strip chart recorder.

Muscle Stimulation

Muscle twitches were evoked by direct electrical stimulation of the muscles near their origins or insertions and by stimulation of the trochlear or hyomandibular nerves. Stimulating currents were passed between a Ringer filled pipette (30 μm i.d.) positioned next to the muscle or nerve and a ground electrode in the bath. Square-wave stimulus pulses of 1 ms duration were used. TTX-free Ringer was used during muscle stimulation experiments.

Anticholinesterase Experiments

AChE activity was blocked by an irreversible anticholinesterase, methanesulphonyl fluoride (MSF) (Kordas *et al.*, 1975). In order to determine the dose of MSF required to

produce maximal inhibition of AChE, we measured its effect on the decay constants of MEPCs recorded at mature myotomal synapes (stages 47-52) where AChE is abundant (Kullberg *et al.*, 1980). The muscle was soaked in different concentrations of MSF for 45 min, followed by 30 min wash out. The dose response data are plotted in Fig. 4. Application of 3 mM MSF produced a maximal prolongation of MEPCs and also completely eliminated staining of AChE by the method of Karvnosky and Roots (1964). Similar application of MSF to the interhyoideus and superior oblique muscles eliminated any AChE detectable by the Karnovsky-Roots stain. In all MSF experiments reported here, the drug was used at 3 mM concentration with the above exposure and wash-out times.

Acetylcholinesterase Histochemistry

We used the method of Karnovsky & Roots (1964) to detect accumulations of AChE at endplates and myotendinous junctions. Stained muscles were examined under 200X or 320X. The following staining procedure was used:

Animals were fixed overnight in cold formalin and 1% Calcium chloride. Before staining, the preparation was rinsed in distilled water or buffer for 5 min. All solutions used in staining were made up in phosphate buffer (pH 6). Stock solutions of 0.1 M sodium citrate, 3 mM copper sulfate and 5 mM potassium ferrocyanide were made in advance. At the time of staining, 10 ml of staining medium were made by adding 5 mg acetylthiocholine iodide to 6.5 ml of buffer, then adding, in order and with stirring, 0.5 ml of 0.1 M sodium citrate, 1.0 ml of 3 mM copper sulfate, 1.0 ml of 5 mM potassium ferrocyanide and 1 ml distilled water. Muscles were stained in this solution for about 1 h, rinsed with buffer and examined under the microscope.

Immobilization Experiments

For the purpose of studying the effects of immobilization on the development of endplate currents, embryos were divided into control and experimental batches and their egg and vitelline membranes were removed before the onset of motor activity. Experimental animals were placed in a bath containing TTX (Sigma) at a concentration of 20 μg/ml or 100 μg/ml. Embryos which were placed in 100 μg/ml were transferred to TTX, 20 μg/ml, after about 15 h. Solutions were changed twice daily. TTX is a potent and specific blocker of voltage gated sodium channels (Kao, 1966; Catterall, 1980). The toxin gains entry to the muscle and nervous system of *Xenopus* embryos and tadpoles directly from the bath. It seems likely that all TTX blockable action potentials were effectively eliminated in experimental embryos. Under these conditions nervous system activity should have been almost completely suppressed and the embryos were probably anesthetized as well as being paralyzed.

Stage	Age	Comments
17	18 3/4hr	late neural fold stage; myotomes begin to form
19	20 3/4hr	4-6 myotomes; neural folds touching
21	22 1/2hr	8-9 myotomes; neural tube completely formed
24	26 1/4hr	15 myotomes; initial motor responses
26	29 1/2hr	17 myotomes; beginning of spontaneous movements
33/34	1d 20hr	beginning of heart beat
35/36	2d 2hr	embryo hatches
50	15d	both fore- and hindlimb buds distinct; hindlimb bud constricted at base

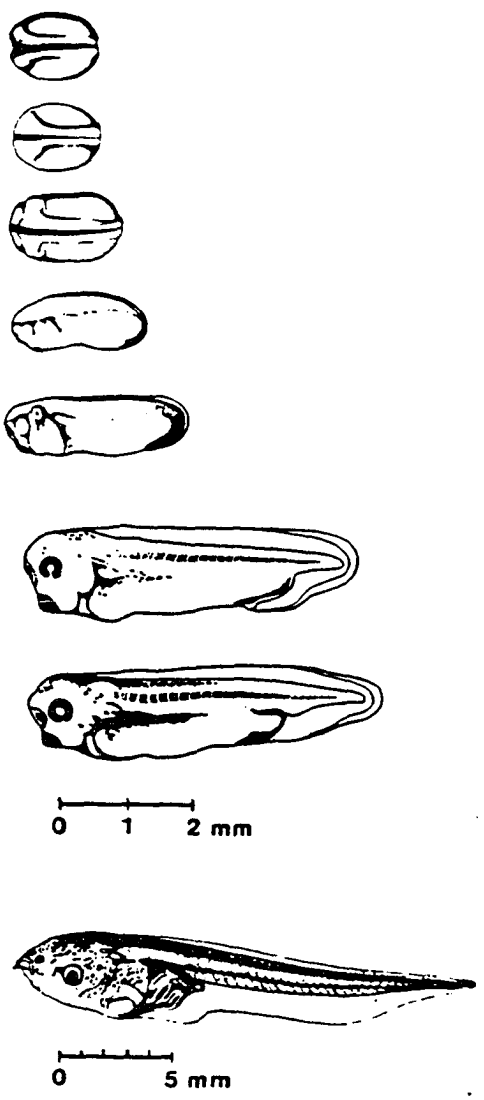

Figure 1. Selected developmental stages of *Xenopus laevis* (from Nieuwkoop and Faber, 1967). The first AChRs appear at stage 19 and initial synaptic activity in myotomal muscle can be detected at stage 21. Reflex movement in response to mechanical stimulation first appears at stage 24, and spontaneous movement begins at stage 26. Stage 33/34 is a landmark stage for the development of postsynaptic activity. At this stage, the high conductance, fast gating AChR channels begin to be expressed abundantly, the low conductance channels undergo a decrease in open time, and the level of AChE is sufficiently high that the decay of synaptic currents is rate limited by AChR channel open time. At stage 50, the myotomal muscle is fully mature.

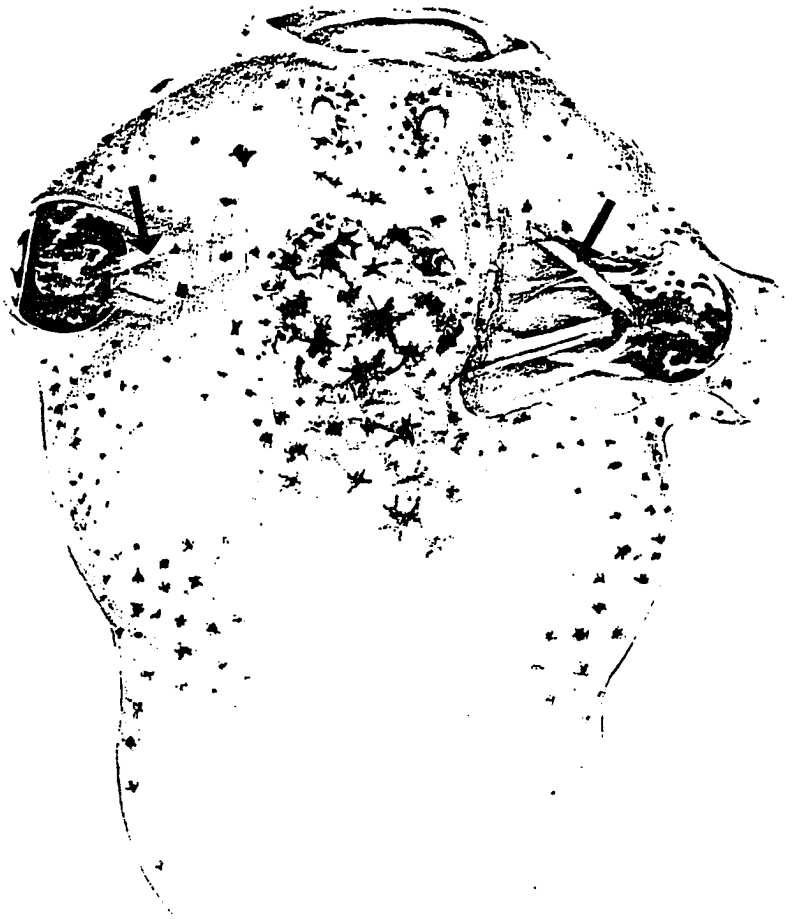

Figure 2. Dorsal view of a stage 47 tadpole illustrating the extraocular muscles. The skin is removed over the muscles on the right side. Arrows indicate the superior oblique muscles.

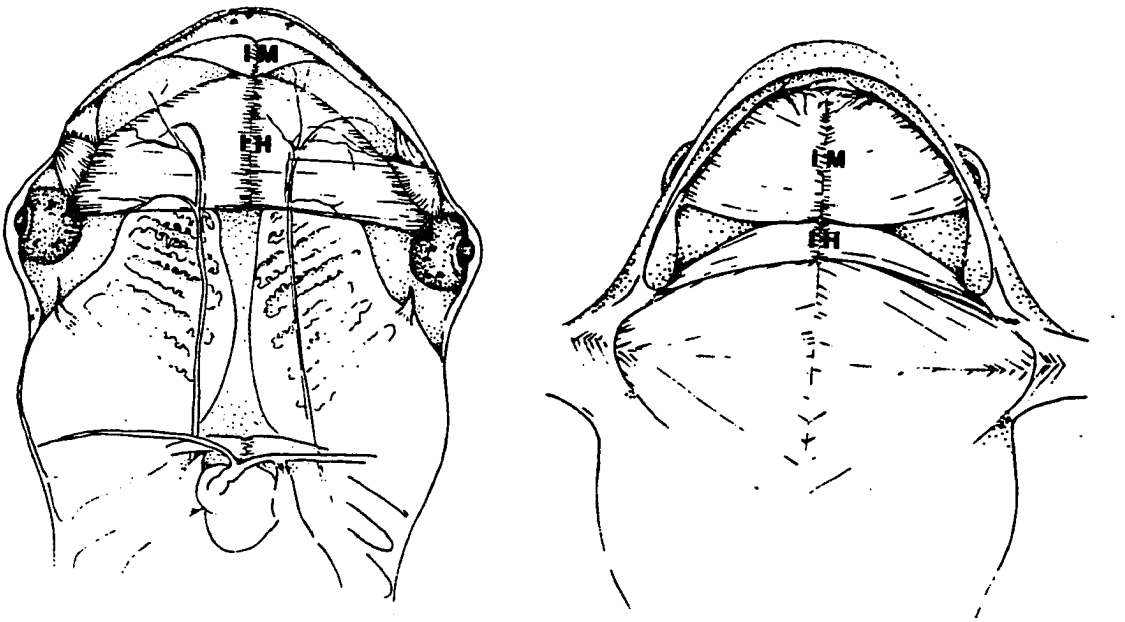

Figure 3. Ventral view of a stage 47 tadpole (left) and a newly metamorphosed frog (right) illustrating the interhyoideus (IH) and intermandibularis (IM) muscles. These drawings illustrate the changes in position and size of the two muscles during development.

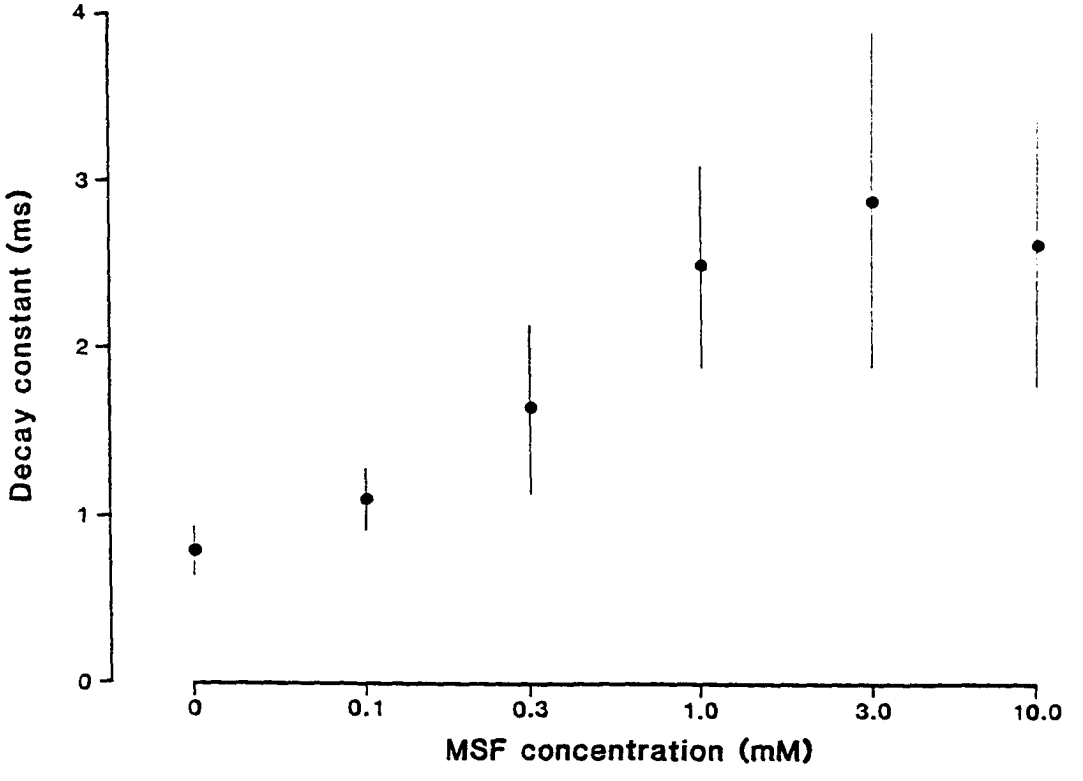

Figure 4: Dose-response data showing the effect of MSF at different concentrations on MEPC decay constants. Each point represents the mean decay constant (± s.d.) of several recording sites. The numbers of recording sites at different concentrations, from left to right, were 38, 12, 9, 15, 24, 15.

RESULTS

Development Of Acetylcholine Receptors

AChR single channel activity was recorded from myotomal muscle in embryos as young as 21 h (stage 19), which is the earliest stage at which nicotinic AChRs have been detected by uptake of radiolabeled α-BTX (Chow & Cohen, 1983) or by depolarization in response to bath applied ACh (Kullberg, 1974). Stage 19 is 2 h prior to the first evidence of innervation (Blackshaw & Warner, 1976a; Kullberg *et al.*, 1977) and 5-6 h before the muscle begins to contract (Nieukoop & Faber, 1967; Kullberg *et al.*, 1977). Records were taken at each stage of development between stages 19-25 (a 6 hour period) and at about every fourth stage over the next 25 days (stage 25-54). The oldest muscle studied was at stage 61 which is 5 days before completion of metamorphosis. The number of recording sites analyzed at each stage ranged from 4 to 14 with an average of 7. Examples of single channel records are shown in Fig. 5. Notice that at early stages only one conductance class is evident, but in older muscle there are two, both of which have briefer mean open times than channels present in early stage muscle.

The frequency and character of single channel currents depended on the age of the muscle. When 500 nM ACh was present in the patch pipette, substantial channel activity was observed at stages 25 to 48 in almost 100% of the patches. In very young stages (19-24) and in mature muscle (stages older than 48) some patches had no identifiable channel activity or else the event frequency was too low for analysis. This variability may reflect a lower density of nonjunctional AChR channels in very young and mature muscle relative to intermediate stages (Chow & Cohen, 1983).

Single channel conductances

When multiple openings were eliminated, the single channel currents fell into one of four amplitude classes. Only the two largest currents were abundant enough for analysis in the majority of our records (102/123 records). Occasionally it was possible to observe all 4 current levels in the same record. In records without overlapping events, openings of each amplitude class occurred in isolation. Rarely were events seen which could be interpreted as transitions from one amplitude class to another. This is in contrast to the partial closure of channels to a subconductance state, which was reported in cultured muscle cells from rat (Hamill & Sakmann, 1981; Takeda & Trautmann, 1984; Sigworth, 1985), mouse (Morris & Montpetit, 1986), chick (Auerbach & Sachs, 1983; 1984) and in adult frog muscle (Colquhoun & Sakmann, 1985). The current-voltage relationships of the smaller two currents indicate slope conductances of 10-15 pS and 20-30 pS. Small, independent events have also been noted in cultured *Xenopus* muscle cells (Brehm *et al.*,

1984b, Auerbach & Lingle, 1986) but were not investigated. Preliminary experiments indicate that the activity of the two smaller channels was blocked by α-BTX , but their identity as ACh activated channel currents is not yet firmly established. For the remainder of this report only the two larger and more frequently encountered channel currents will be described.

The two major classes of ACh receptor channels we found during development resemble those previously reported in cultured *Xenopus* myocytes (Leonard et al., 1984; Brehm *et al.*, 1984b; Greenberg *et al.*, 1985; Auerbach & Lingle, 1986), mature myotomal muscle (Brehm *et al.*, 1984a), rat myocytes in culture (Siegelbaum *et al.*, 1984) and developing rat (Schuetze *et al.*, 1985; Vicini & Schuetze, 1985) and bovine skeletal muscle (Mishina *et al.*, 1986). In keeping with previous nomenclature and for the sake of convenience we will refer to the two conductance classes we observed as "40 pS" (or "small") and "60 pS" (or "large") channels. Currents belonging to the two conductance classes are shown in Fig. 5. Based on the cumulative data the single channel current amplitude at resting potential was 3.3 ±0.6 pA (n=80) and 4.6 ± 0.8 pA (n=42) for the two channel types. The amplitudes of these events differed by a factor of about 1.4 at all applied potentials, which indicates that the larger events were not the sum of smaller currents.

As reported previously (Brehm *et al.*, 1984a,b; Auerbach & Lingle, 1986) the current-voltage (I-V) relationships of both the 40 pS and 60 pS channels were non-linear (Fig. 6). The slope conductances of both classes increased with membrane hyperpolarization. To further describe the dependence of conductance on membrane potential, we measured the slope conductance between successive 20 mV steps of applied potential between 0 and 80 mV. When the mean slope conductance was plotted against the corresponding change in potential (Fig. 7), a linear relationship for both channels was observed. The lines of best fit to these data gave gave the following equations for the 40 and 60 pS channels:

$$\gamma_{40} = 33.40 + 0.16V_p$$

$$\gamma_{60} = 43.30 + 0.38V_p$$

where V_p = applied pipette potential (in mV)
and γ_{40} = channel conductance (in pS) of the small channel
γ_{60} = channel conductance (in pS) of the large channel.

At an applied potential of 40 mV, where the recordings were most commonly made because of the improved signal to noise ratio, the two slope conductances were 39.8 pS and 58.5 pS.

There was no change in the I-V relationship of either channel during development. For instance, the slope conductances of the 40 pS channel were described by $\gamma_{40} = 33.9 + 0.15V_p$ from stages 19 to 30, and by $\gamma_{60} = 32.8 + 0.17V_p$ from stages 33 to 61.

Reversal potentials

The reversal potentials for the two channel types were difficult to measure with confidence. Extrapolation of the I-V relationship to the zero current level did not give a valid estimate because of the nonlinearity just mentioned. A further complication resulted from the voltage sensitivity in the closing rate of both channels (discussed below). When the membrane was depolarized until currents disappeared or reversed, the channel open time became so rapid that most events were attenuated by the recording system. Nevertheless, we obtained a rough estimate of the reversal potentials by adjusting the applied pipette potential until the currents disappeared. The channel currents disappeared at -70 to -80 mV (n=11) applied potential. Assuming a resting membrane potential of -70 to -80 mV (see below), the zero current level occurs at a membrane potential near 0 mV, which is comparable to the reversal potentials previously reported for ACh channels (Brehm *et al.*, 1984 a,b).

Developmental changes in conductance classes

During early developmental stages (19 to 30), the predominant channel openings belonged to the 40 pS class. Out of 71 recording sites in these stages, only 5 had evidence of significant 60 pS channel activity. However, by stage 33 more than 60% of the recordings contained high conductance channels, and thereafter this fraction progressively increased to 100% in mature muscle. Along with an increase in the percentage of cells expressing high conductance channels, the relative frequency of 60 pS events at individual recording sites also increased as the muscle developed. In Fig. 8 current amplitude histograms illustrate the changing prevalence of the two channel types at selected stages of development. The relative increase in frequency of high conductance channel openings at all recording sites is shown in Fig. 9. From these data it is obvious that the major period of transition in channel types occurs between about stage 33 and stage 50. Several recordings from muscle older than about one month (about stage 54) contained only the high conductance channel. Our estimates of relative event frequency were based on the number of observed channel openings assigned to each class. Because a large number of events were missed, due to the frequency response of the recording system, these data do not represent either the relative numbers of channel types actually present in the patch or an accurate description of their relative

activity. Nevertheless, these observations support the general conclusion that large channels progressively outnumber the smaller ones during development of myotomal muscle and that this change largely occurs following stage 33.

Developmental changes in gating kinetics

We analyzed all channel openings which were at least 300 μs in duration and were separated by gaps of at least 200 μs from neighboring events. Openings briefer than 300 μs in duration had a wide amplitude variance which prevented their unambiguous assignment to amplitude classes. Open duration histograms were compiled at applied potentials ranging from 0 to 100 mV. The apparent mean channel open times (τ) were estimated from these histograms by the method of maximum likelihood (Colquhoun & Sigworth, 1983).

40 pS channels:
At the resting membrane potential (0 mV applied potential) more than 90% (46/51) of the open duration histograms at all developmental stages were satisfactorily fitted by single exponential curves. The estimated open time of the 40 pS channels became briefer during development (Fig. 10 & Fig. 11), in agreement with previous observations made *in vitro* (Leonard *et al.*, 1984). The open time remained fairly constant throughout the early stages of development and appeared to undergo a transition to a briefer open time at about stage 33 (Fig. 11). At stages 19-30, the mean channel open time with no applied potential was 2.8 ± 0.9 ms (n=18) as compared to 1.6 ± 0.7 ms (n=28) at stages 33 to 61. The voltage dependency of the open time did not change significantly during development, although there was a wide variance in the data and small differences would not have been noticed. The open time increased e-fold with 70-95 mV hyperpolarization at all stages of development (Fig. 12)

In view of this voltage dependency, it is unlikely that a decrease in resting potential was responsible for the nearly 2-fold reduction in open time we observed during development. The observed change in open time would require approximately a 40 mV reduction in resting membrane voltage, but previous work has shown that the myotomal membrane potentials are not measurably diminished during development (Blackshaw & Warner, 1976b; Kullberg *et al.*, 1977). The similarity of current amplitude during development further suggests that there was no major change in resting potential of the cells. At 40 mV applied potential, the 40 pS single channel currents were 4.3 ± 1.0 pA, (n=48) at stage 19-30 and 4.7 ± 0.9 pA, (n=36) at stage 33-61.

60 pS Channels:
All 60 pS channel open duration histograms at resting membrane potential were well fitted by single exponential functions (Fig. 10). The mean open time of the 60 pS

channel at the resting potential was 0.8 ± 0.4 (n=34). The open time was prolonged by e-fold with 50-60 mV hyperpolarization (Fig. 12). Neither the channel open time nor its voltage dependence was noticeably altered during development. A few histograms deviated from a single exponential at applied potentials of 60 mV or more but will not be considered further here.

Control experiments

In order to determine which channel activity was elicited by ACh we recorded from muscle without agonist present in the patch pipette. Precautions were taken to ensure that no ACh remained in the recording dish or on the silver chloride wire electrode. In some recordings, no currents resembling those from the 40 pS or 60 pS channels were seen, but in others, channel activity occurred which was similar to that observed in the presence of agonist. In those records where channel activity persisted in the absence of agonist, the event frequency was usually much higher as the seal formed and declined dramatically over the next minute to <1 event s^{-1}. The initial frequency of events was usually increased if prolonged suction was used to establish the seal and the single channel currents that resulted were often rounded in appearance. This problem was encountered more frequently in muscle which had been treated with collagenase prior to recording. Others (Brehm *et al.* 1984a,b; Morris & Montpetit, 1986) found similar AChR-like channel activity in the absence of agonist, and Jackson *et al.* (1984) reported that AChR channels can open spontaneously.

In further control experiments we examined the effects of α-BTX on channel activity. This toxin is a highly specific and essentially irreversible blocker of nicotinic AChRs on skeletal muscle (Lee, 1979). In two sets of experiments we determined which single channel currents were abolished by a saturating dose (Brehm *et al.*, 1983) of α-BTX. These experiments were done on muscle which allowed rapid and stable seal formation without enzyme treatment and consistently exhibited substantial channel activity when ACh was present.

In the first set of experiments, 5 muscles were bathed for 15 min in α-BTX (0.1 mg/ml) and 31 recordings with no agonist in the pipette were then obtained. Each recording was maintained at least 1.5 min with an average of 2.4 min. During the recording period the patch was hyperpolarized 60 to 80 mV from rest. During a total recording time of 75 min we saw no 40 pS or 60 pS channel activity, and in 75% of the records (23 out of 31) the patch was completely silent. The lack of activity was not due to failure to maintain cell attached patches nor to depolarization of the muscle cells, because at the end of four recordings we were able to gain entry to the cell's interior by applying suction and estimated the resting potential to be -97 ± 12 mV (not corrected for diffusion

potentials). Furthermore, it was possible to record ACh activated channel openings, presumably from newly inserted AChRs, in each of 3 of the muscles after the toxin was washed out and they were allowed to recover for 1 to 3 h.

In a second set of experiments we added 1.0 μM ACh and 0.1 mg/ml α-BTX to the recording pipette to determine directly which channel activity was originally present before it was eliminated by the toxin. In the presence of the toxin, channel activity often disappeared so rapidly that it was not possible to identify the events according to their slope conductance. However, in 39 recordings we collected enough events to identify which conductance classes were present in the patch before they were abolished by the toxin. 40 pS and 60 pS channel activity typically persisted for <4 min before completely disappearing. At the end of some recordings, after a long period of toxin induced silence we were able to verify that the patch was still cell-attached by breaking the cell membrane with negative pressure and observing spontaneous synaptic currents. In records taken from the same muscles but with ACh and no toxin in the pipette event frequency remained high for the duration of the recordings, which was over 12 min in some cases.

These results demonstrate that the two major conductance classes of events we commonly observed in our single channel recordings were blocked by α-BTX, which is strong evidence that they are due to ACh receptor openings (Lee, 1979).

Suction-activated channels

Suction-activated channels have been previously reported in *Xenopus* muscle *in vivo* (Brehm *et al.*, 1984a) and *in vitro* (Brehm *et al.*, 1984a,b) and other membranes (reviewed by Kullberg, 1987). In the control experiments described above we routinely applied suction to the patch after channel activity appeared to be completely blocked by the toxin. In a small fraction of the patches (<20%) suction elicited rapid bursts of openings with many unresolved closures. These channels exhibited a discrete activation and inactivation when suction was applied and released. Unlike the toxin-blockable channels, there was no noticeable change in kinetics of these channels associated with increased hyperpolarization of the patch. The voltage-insensitivity and toxin resistance suggest that the channels activated by suction are not AChRs. However, the similarity of current amplitudes between suction-activated and AChR channels could result in confusion of the two. Therefore in all recordings with agonist in the pipette, we routinely tested for suction-activated channels. Records showing such activity were not analyzed.

Summary of results

1. The development of ACh receptor channel function in *Xenopus* myotomal muscle was studied by the method of single channel recording. AChR channels were studied from the time of their first appearance on the muscle membrane until the time of full maturity of the muscle.

2. Two conductance classes of AChR channels were detected. The slope conductance of each class increased linearly with hyperpolarization over a range of 0 to 80 mV. At rest, the conductances were 33 and 45 pS; at 40 mV, they were 39 and 58 pS.

3. During the first day after their initial appearance on the membrane, the small conductance channel was the predominant form on the muscle membrane. The large conductance channel then began to be expressed in significant numbers and over the next two days became the dominant channel type.

4. The large conductance class had a mean open time of 0.8 ms, which remained constant throughout development. The small channel initially had an open time of 2.8 ms, which later decreased by almost 50% to 1.6 ms. The change in open time of the small channel was correlated with the onset of expression of the large conductance channels, suggesting a possible coregulation of the two channel types.

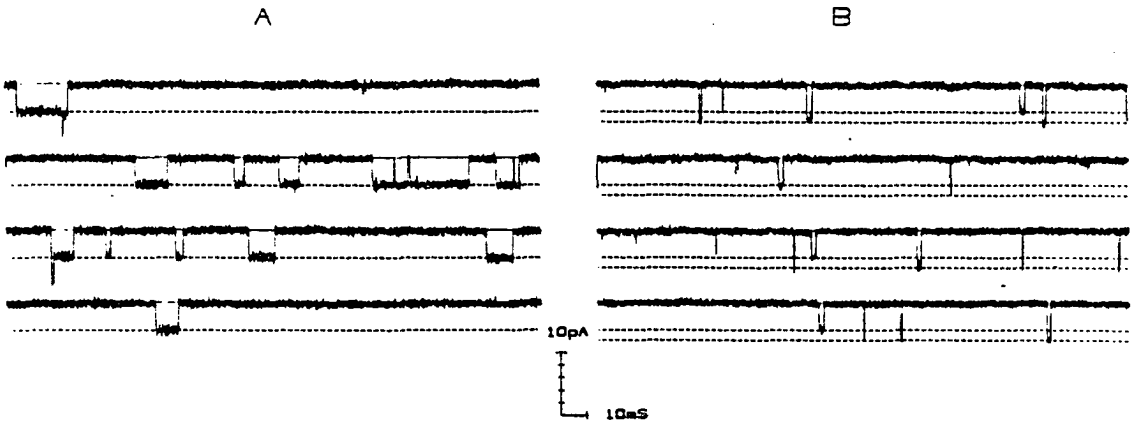

Figure 5. Examples of single ACh receptor channel openings recorded from nonjunctional regions of early and intermediate stage myotomal muscle. Recordings were made at 40 mV applied pipette potential from cell-attached patches. Each panel shows consecutive traces. Recordings from stage 24 muscle are shown in the left panel (A). Only one conductance level (indicated by dotted line) was evident. Records from stage 39 muscle are shown in the right panel (B). Two conductance levels were evident and the open durations of both conductance classes were less than that of the early stage muscle.

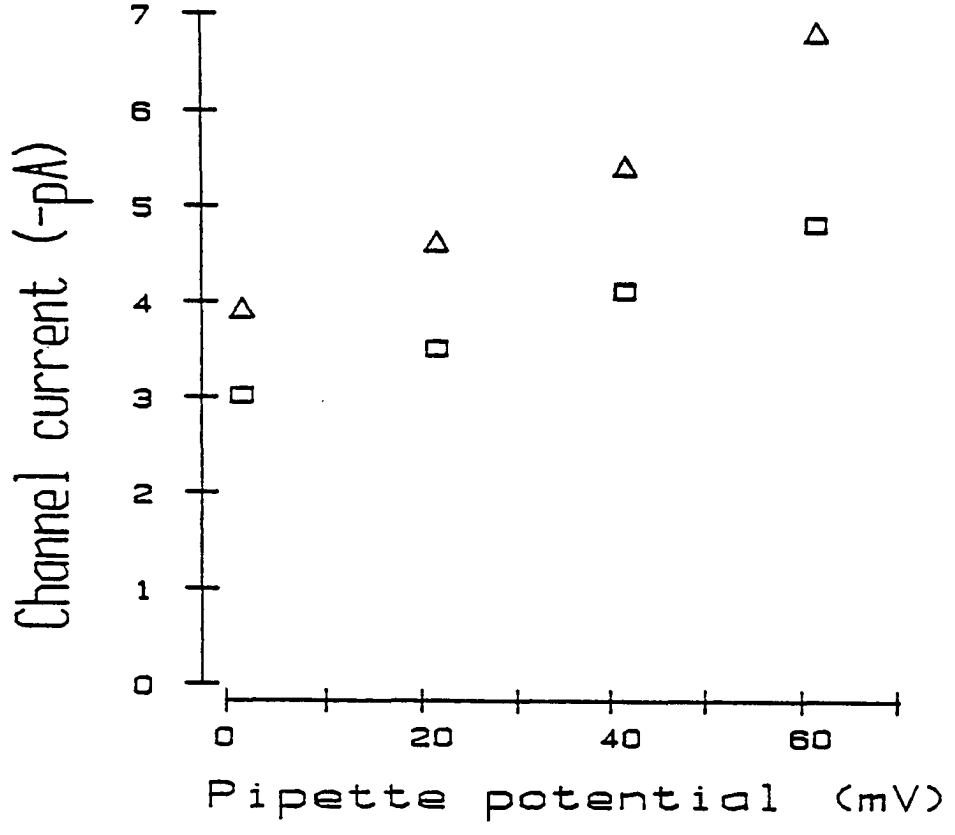

Figure 6. Single channel current from low and high conductance channels vs. applied pipette potential. Channel currents are indicated by triangles (large channels) and squares (small channel). These data are taken from a single recording site at stage 40. The nonlinearity of current vs. voltage is evident in both channel types.

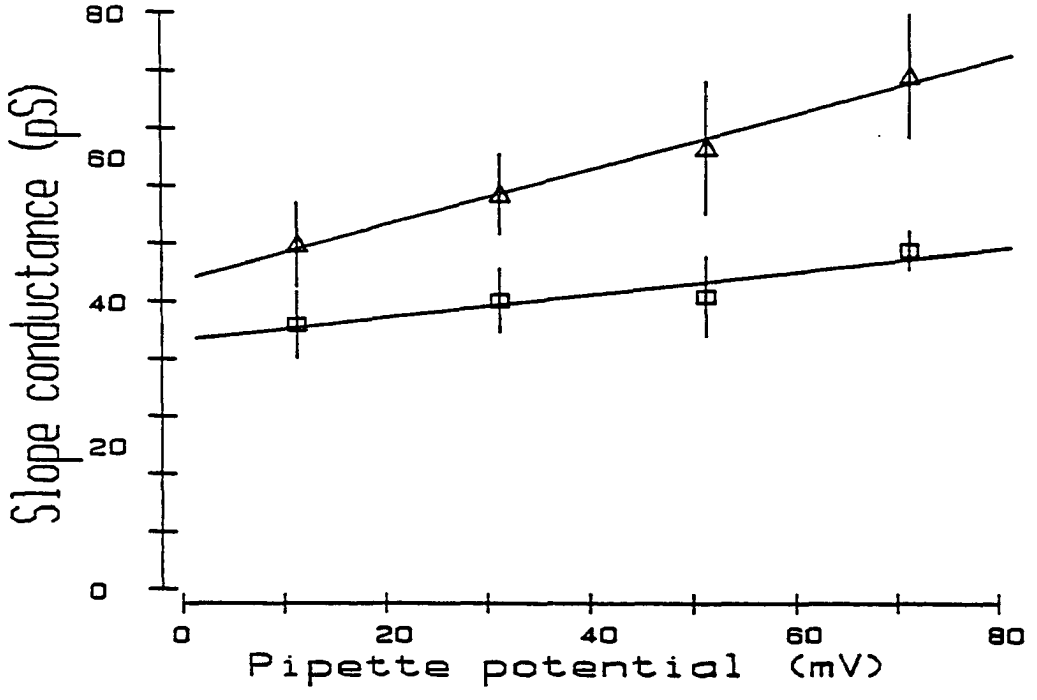

Figure 7. Mean slope conductance vs. applied potential for large (triangles) and small (squares) channels. Bars indicate standard deviations. These values are based on the cumulative data from all recording sites throughout development. Recordings were taken at 0, 20, 40, 60 and 80 mV applied potential. The slope conductances between adjacent 20 mV applied potentials were calculated at each recording site and then averaged to obtain the mean values plotted in this figure. The mean slope conductances are plotted midway between each 20 mV level. The lines of best fit were calculated by linear regression and the parameters of each line are given in the text.

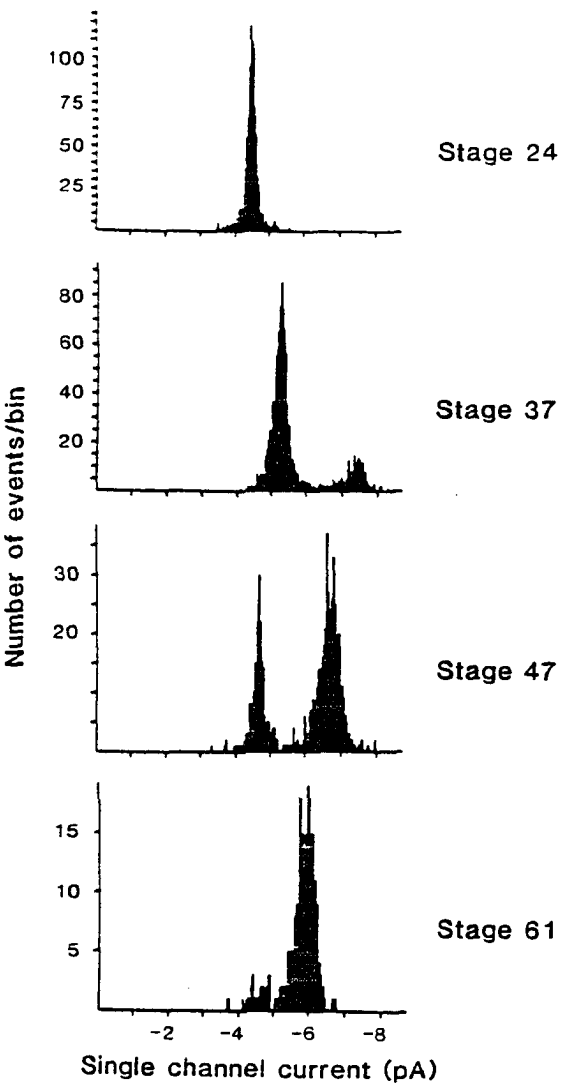

Figure 8. Amplitude histograms of single channel currents at 40 mV applied potential. These histograms reveal two amplitude classes of channels and demonstrate the increasing prevalence of the large amplitude events with development.

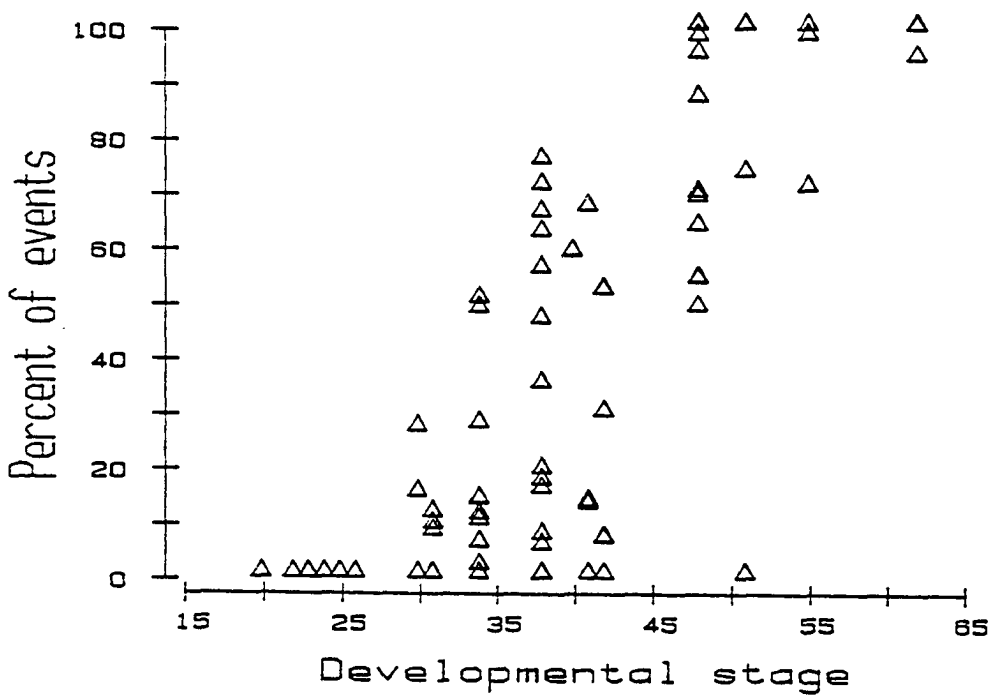

Figure 9. Percentage of large conductance events vs. developmental stage. This figure demonstrates the increasing frequency of large conductance events during development. Each data point represents the percentage of total events which belonged to the large conductance class of AChRs. In most cases, data points at 0% and 100% represent multiple recording sites (up to 8) at a single stage. The data points from stages 19 to 25 represent a total of 48 recording sites at which negligible large channel activity was detected. All other data points represent single recording sites.

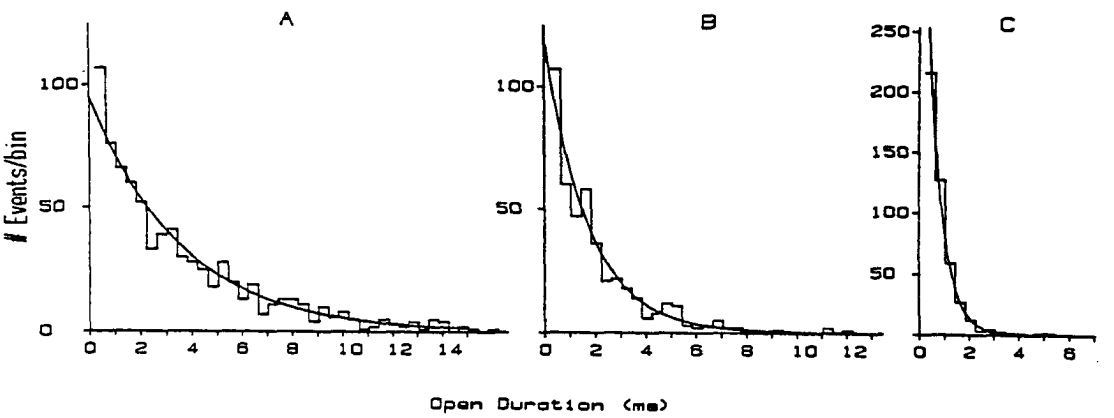

Figure 10. Open duration histograms of ACh receptor channels at different developmental stages. A. Stage 24. B. Stage 41. C. Stage 50. These histograms illustrate the decrease in channel open time which occurs during development of myotomal muscle. The histograms were compiled from single openings which were separated by gaps of 0.2 ms or more from neighboring events. The recordings were made at 0 mV applied potential from nonjunctional regions of myotomal muscle. Each histogram is fitted by the maximum likelihood estimate of a single exponential function. Note that the histograms at stages 24 and 41 correspond to the low conductance channel openings and the histogram for stage 50 corresponds to openings by the high conductance channel.

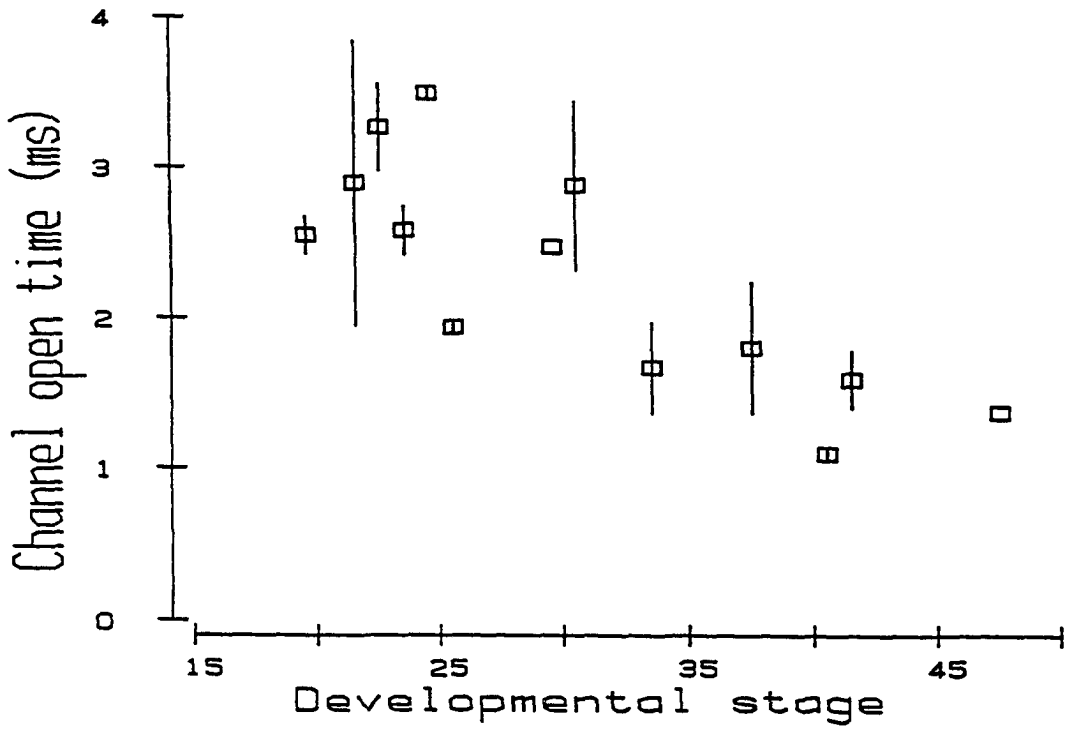

Figure 11. Channel open time (± s.d.) of small channels vs. developmental stage. Each data point represents the mean ± s.d. of all recordings at a single stage. The average sample size at each stage was 4. Symbols without bars indicate single recording sites. This figure demonstrates the decrease in open time of the small channel which takes place during development.

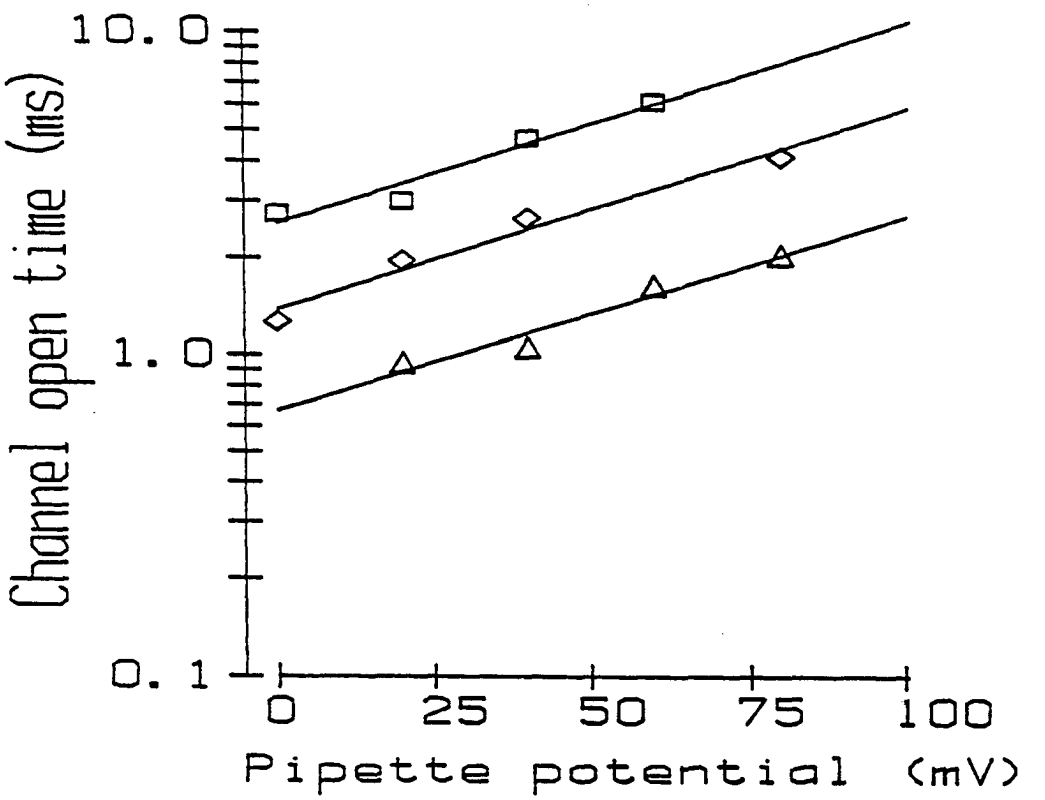

Figure 12. Mean channel open time vs. applied pipette potential for three selected records from different kinetic classes of channels. Triangles, large conductance channels at stage 54; diamonds, small conductance channels at stage 33; squares, small conductance channels at stage 23. These records further exemplify the differences between mean open time of the small channel at early and intermediate stages of development. All three kinetic classes exhibited an increasing open time with hyperpolarization. The average changes in applied potential necessary to produce an e-fold increase in open time for the different conductance/kinetic classes of channels were as follows: large channels (stages 33-62), 63.3 ± 31.6 mV (n=43); small, slow channels (stages 19-30), 94.1 ± 71.3 mV (n=50); small, fast channels (stages 33-62), 72.8 ± 36.2 mV (n=41).

Development of Endplate Currents in Immobilized Muscle

Effects of tetrodotoxin on motor activity

To study the effects of immobilization on the development of endplate currents, *Xenopus laevis* embryos and tadpoles were reared in the presence of a paralytic toxin, TTX. When hatched embryos or tadpoles were placed in water containing TTX, their spontaneous motor activity gradually ceased and they became unresponsive to mechanical stimulation. Once immobilized, even the most vigorous stimulation failed to produce any twitch response of the muscle. It was noted that occasionally a slow contracture lasting 1 to 2 min could be produced by strongly prodding the myotomes. Such slow contractures were never seen spontaneously. In contrast to the TTX-treated embryos and tadpoles, the control animals were responsive to the slightest touch and were spontaneously active from stage 26 on. Older animals were more readily affected by TTX than younger. Stage 41 tadpoles, when placed in 20 μg/ml TTX became immobile within 6 min, whereas stage 26 embryos required up to 6 h to become completely unresponsive to mechanical stimulation. Elevating the TTX concentration to 100 μg/ml eliminated movement in stage 26 embryos within 2 h. In most experiments, we removed embryos from their eggs at stages 22-23 and placed them immediately in 20 μg/ml TTX. Although it seems unlikely that any significant amount of muscle activity occurred in embryos raised in TTX from stages 22-23 on, it was important to rule out any possible movement at early stages. Therefore, in two experiments, we hatched embryos at stages 16 to 18, which is 6 to 8 h before the onset of motor responsiveness (stage 24) and placed them immediately in 100 μg/ml TTX. Since most of these embryos were slightly injured during the hatching process, due to the difficulty of removing the vitelline membrane, it seems likely that the TTX gained rapid entry into the muscle. Those embryos which healed quickly and developed normally were used for recordings. At stages 28-30 they were transferred to 20 μg/ml TTX. Experimental results obtained from these animals were similar to those obtained from animals hatched at stages 22-23 and raised in 20 μg/ml TTX.

The morphology of TTX-reared embryos and tadpoles appeared entirely normal up to about stage 41. After that time, they gradually became edematous. The swelling was considerable in the abdominal and cloacal areas, but none was evident in the region of the myotomal muscle. Following the onset of edema, tadpoles were no longer able to recover from immobilization when placed in TTX-free water. Whether this represented a retention of TTX or a different process is unclear. It was possible to estimate the developmental stage of the tadpoles up to stage 45 and occasionally stage 46 on the basis of intestinal shape, however the gut began to shrivel at that time (feeding normally

begins about stage 45), and we could not confidently stage older tadpoles. Despite the edema, the myotomal muscle remained healthy, as judged by its appearance, the presence of normal MEPCs and appropriate resting potentials. Intracellular recordings showed membrane potentials of 79 ± 7 mV (n=10) in control and 71 ± 8 mV (n=24) in TTX-reared tadpoles at stages 45-46.

Miniature endplate current rise times

Exposure of developing muscle to TTX did not affect the developmental change in rise time of focal MEPCs (Fig. 13). At stages 24-26, rise times of MEPCs were 2.9 ± 0.7 ms (n=32) in control embryos and 2.8 ± 0.8 ms (n=10) in embryos raised in TTX. By stage 32, rise times in both control and TTX-reared embryos had decreased to about 1 ms. There was a gradual further reduction through stages 44-46 at which age the rise times were 0.6 ± 0.1 ms (n=44) in controls and 0.6 ± 0.1 ms (n=81) in TTX-reared tadpoles. We did not obtain recordings of MEPCs beyond stage 46 in TTX-reared animals, but at this stage of development the rise times had nearly achieved their mature value of 0.4 ± 0.1 ms (n=41), as recorded from stage 47-50 control tadpoles.

Miniature endplate current decay constants

The decay phases of MEPCs were of three kinds: single exponential, double exponential, and nonexponential. At all developmental stages, MEPCs were recorded which appeared to be well-fitted by single exponential decays (Fig. 14). Fig. 14 demonstrates the similarity of MEPCs from control and immobilized muscle throughout the period of development we studied. There was a significant decrease in the decay time of these MEPCs in both control and immobilized muscle during development (Fig. 15). In control embryos the decay constants at stages 24-26 were 6.8 ± 2.4 ms (n=32), as compared to 7.5 ± 3.0 ms (n=10) in TTX-bathed embryos. As observed for rise times, the greatest rate of change in decay constant occurred during the first 10 h or so following onset of motor responsiveness. By stage 30, the decay constants had decreased to about 3 ms in both control and TTX-reared embryos. A more gradual decrease occurred during the following 60 h. At stages 44-46, the decay constants of single exponential MEPCs were 1.5 ± 0.5 ms (n=44) in control and 1.2 ± 0.5 ms (n=81) in TTX-reared tadpoles. Non-exponential MEPCs were seen predominantly in the youngest embryos (less than stage 28). They tended to have a plateau following the rising phase or else they decayed more slowly than a single exponential function, possibly reflecting a response of voltage-gated channels to the synaptic depolarization. These MEPCs were not analyzed further.

Double exponential MEPCs (Fig. 16) were first seen at stage 33/34. It is about this stage that ACh receptor channels with rapid gating kinetics are first revealed by spectral analysis of ACh noise (Kullberg & Kasprzak, 1985) and by single channel recordings as described above. Both single and double exponential MEPCs were seen at individual recording sites at all subsequent stages. No site at any stage had exclusively double exponential MEPCs. Initially, about a third of the recording sites had MEPCs with double exponential decays. The frequency of such sites increased with development, reaching a peak of more than 80% at stages 43-44. At stages 45 and 46 the frequency of sites with double exponential MEPCs decreased to about 50%. Control and TTX-reared tadpoles were comparable with respect to the values of the fast and slow time constants of double exponential MEPCs (Figs. 16 & 17). The fast components were about 1.5 ms at stage 33/34 and declined to less than one ms beyond stage 38. The mean fast component at stages 44-46 was 0.7 ± 0.2 ms (n=26) in control and 0.7 ± 0.2 (n=49) in TTX-reared tadpoles. There was relatively more scatter in the estimates of the slow component, but there also appeared to be a decline in the time constant of decay with development. Initial values were about 4 ms at stages 33/34 to 40 and declined to somewhat less than 3 ms at later stages. At stages 44- 46, the mean slow component was 2.9 ± 0.9 ms (n=26) in control tadpoles and 2.8 ± 0.8 ms (n=49) in TTX-reared tadpoles.

When double exponential MEPCs were observed, the contributions of the fast component ranged from 30% to 70% of the amplitude. This percentage did not change noticeably with development and probably reflects the limits of our ability to resolve the two components visually. It seems likely that many of our single exponential MEPCs apparently had two components with one largely outweighing the other.

Effect of anticholinesterase on miniature endplate current duration

The decrease in MEPC duration in immobilized muscle suggested that AChE development proceeded normally in the absence of muscle activity. To verify that AChE was functioning equivalently in immobilized and control muscle, we measured the time course of MEPCs following treatment with the irreversible anticholinesterase, methanesulphonyl fluoride (MSF). The muscle was bathed for 45 min in 3 mM MSF and then washed 30 min in Ringers solution. These conditions produce maximal prolongation of synaptic currents in mature myotomal muscle (Fig. 4). The results of these experiments are summarized in Table 1. MEPCs recorded from immobilized and control muscle had similar durations following block of AChE. Since the MEPC time courses were also comparable before MSF treatment, we conclude that the contribution of AChE toward limiting the duration of transmitter action was similar in control and immobilized muscle. Table 1 shows data for single exponential MEPCs only; however, double exponential MEPCs were also observed in MSF-treated muscle and had comparable durations in both groups. In stage 45-46 control animals the fast and slow decay

constants were 1.7 ± 0.7 and 7.6 ± 1.3 ms (n=7), while in TTX-reared animals they were 1.5 ± 0.7 and 9.6 ± 2.5 (n=12). The difference between the slow components is not significant (P >0.05, two-sided *t* test).

Table 1

MEPC time course following MSF treatment

	Rise time (ms)	Decay constant (ms)	n
Stage 32			
Control	3.8 ± 0.6	7.6 ± 1.6	8
TTX-reared	3.7 ± 0.7	7.3 ± 1.0	4
Stage 45-46			
Control	1.3 ± 0.3	4.9 ± 1.5	16
TTX-reared	1.3 ± 0.2	5.0 ± 1.8	22

Summary of results

1. The effect of chronic immobilization on the development of synaptic currents was studied in myotomal muscle of *Xenopus laevis*. Embryos and tadpoles were immobilized by rearing them in the presence of TTX after removal of the egg membranes.

2. Immobilization did not affect the developmental change in duration of MEPCs. Rise times decreased from about 3 ms to 0.6 ms in both immobilized and control muscle, and decay constants decreased from about 7 ms to 1 - 2 ms in both conditions.

3. MEPCs with double exponential decays were recorded in both immobilized and control muscle at intermediate and late developmental stages. The fast and slow decay constants were 0.7 ms and slightly less than 3 ms in older muscle of both groups. These values are comparable to the apparent open times of fast and slow ACh receptors present on *Xenopus* muscle.

5. Application of an anticholinesterase (MSF) lengthened the duration of MEPCs comparably in immobilized and control muscle.

6. These data indicate that the deposition of junctional AChE and the reduction in open time of AChR channels in developing *Xenopus* myotomal muscle are independent of contractile activity of muscle and TTX-blockable action potentials in muscle or motor neurons.

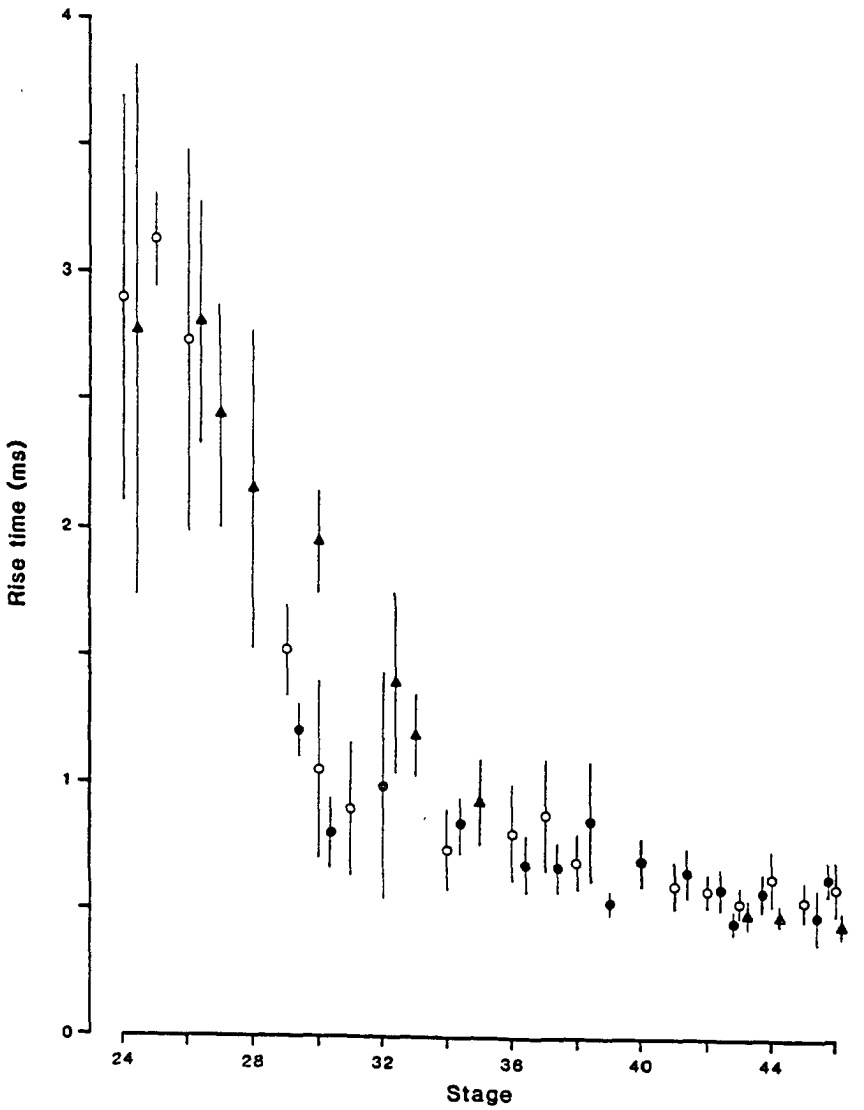

Figure 13: Mean rise times of MEPCs at different developmental stages. Each symbol represents the mean rise time of all recording sites at a single stage of development. The average sample size for each data point is 11 recording sites (range: 3 to 38). Open circles = control; filled circles = TTX-reared (20 μg/ml); filled triangles = TTX-reared (100 μg/ml). Bars indicate standard deviations.

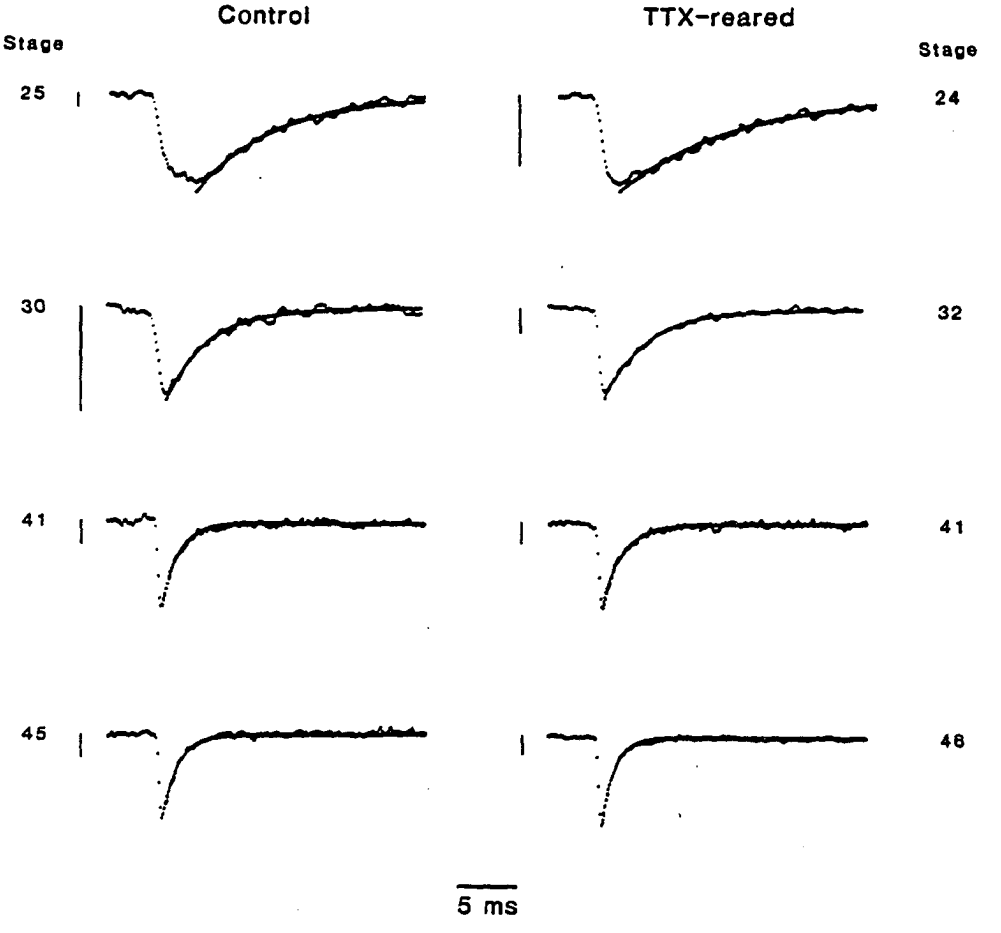

Figure 14: Examples of MEPCs recorded at different developmental stages from control (left) and TTX-reared (right) animals. The declining phase of each MEPC has been fitted by a single exponential function. The decay constants for control MEPCs from top to bottom are (ms) 6.1, 3.3, 1.3 and 1.3. The decay constants from the corresponding MEPCs of TTX-reared animals are (ms) 9.2, 3.5, 1.7 and 1.1. Vertical bars indicate 0.1 mV.

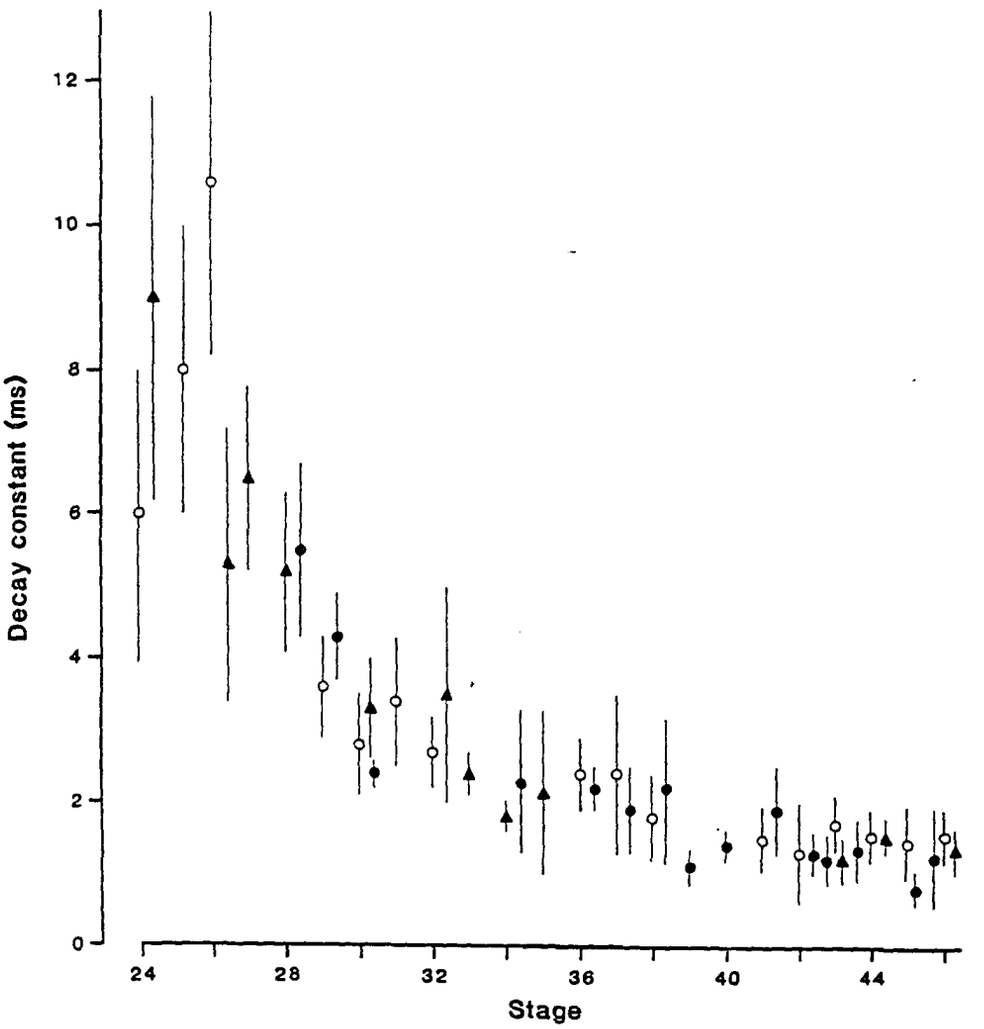

Figure 15. Mean decay constants of single exponential MEPCs at different developmental stages. Each symbol represents the mean decay constant obtained from all recording sites at a single stage of development. The average sample size for each data point is 11 recording sites (range = 3 to 38). Open circles = control; filled circles = TTX-reared (20 μg/ml); filled triangles = TTX reared (100 μg/ml). Bars indicate standard deviations.

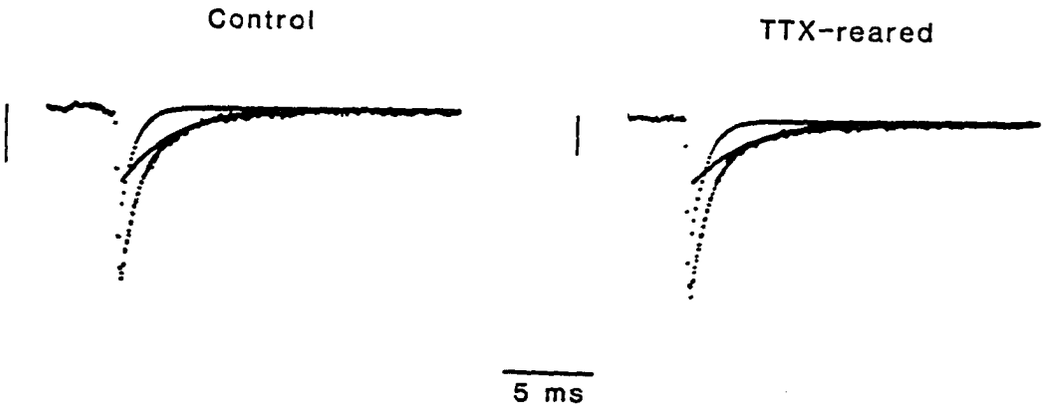

Figure 16. Examples of double exponential MEPCs recorded from control and TTX-reared animals, at stage 41. Each MEPC is fitted by the sum of two single exponential functions. The slow and fast components are displayed as well as their sums, which closely overlap the data points. The decay constants are as follows: Control, 0.8 and 3.0 ms; TTX-reared, 0.8 and 3.1 ms. Vertical bars indicate 0.1 mV.

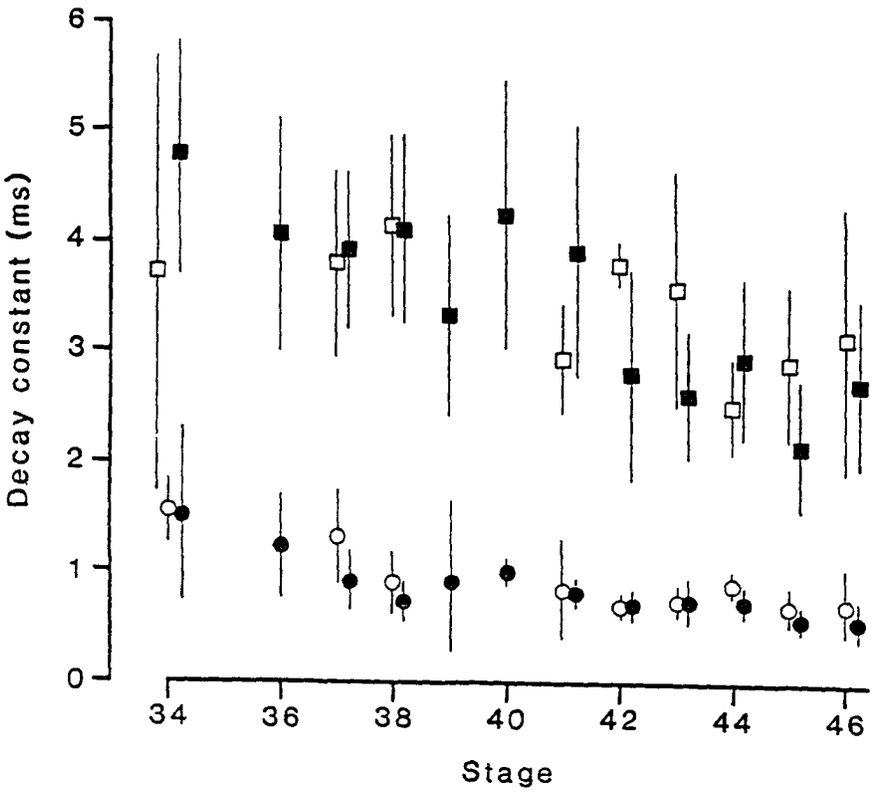

Figure 17. Mean slow and fast decay constants of double exponential MEPCs at different developmental stages. Each symbol represents the mean fast or slow decay constant obtained from all recording sites at a single stage of development. The average sample size for each data point is 8 recording sites (range = 2 to 33). Open symbols = control data; filled symbols = data from TTX-reared animals. Squares = slow decay constants; circles = fast decay constants.

Comparative Development of Endplate Currents

To determine whether the development of postsynaptic function is dependent on muscle type, the development of endplate currents was compared in two functionally different muscles, the superior oblique and the interhyoideus.

Onset of synaptic activity

The earliest stage at which we were able to detect spontaneous synaptic activity or movement in either the superior oblique or interhyoideus was stage 41 or approximately 3 days after fertilization of the egg. Both muscles first became anatomically distinct at about stage 39, which was 20 hours before the earliest detected synaptic activity. Examples of MEPCs recorded at stage 41 and at later stages in each muscle are shown in Fig. 18.

Miniature endplate current development in superior oblique

The earliest MEPCs recorded in the superior oblique had predominantly single exponential decay phases with a mean time constant of 3.3 ms (Fig. 19, Table 2). There was a broad distribution of time constants, ranging from 1 to 9 ms (Fig. 20). A small number of MEPC decays clearly deviated from a single exponential fit (Fig. 21). These could be satisfactorily fitted by the sum of two exponential curves having average time constants of 1.2 and 4.2 ms (Table 2). Such decays, which may have resulted from two classes of AChRs with different gating times, were apparent in about 5% of the MEPCs analyzed at stage 41.

We found no evidence of AChE activity in the stage 41 superior oblique. Karnovsky-Roots stain did not reveal any localized accumulations of AChE, and application of MSF under conditions which should entirely block AChE (Fig. 4) did not lengthen the time course of MEPCs (Table 2).

We did not directly estimate the AChR channel gating times at the newly formed endplates in superior oblique. However, the MEPCs recorded at this stage had decay constants which were comparable to those observed in mature myotomal muscle after block of AChE (Fig. 4), and in the mature myotomal muscle single channel recordings indicate that the majority of AChR channels have gating times of less than 1 ms (See above). This suggests that channels present at stage 41 in the superior oblique were predominantly fast.

MEPC durations became briefer during the first several hours of synapse development. Within 11 hours, the decay constants of single exponential MEPCs decreased to an average value of 1.0 ms and remained there during the following 6 weeks of development (Fig. 19, Table 2). The distribution of decay constants throughout this intermediate period of development had less variability than at earlier or later stages (Fig. 20).

The early decline in MEPC duration was probably due in part to the development of AChE. Whereas we detected no AChE in stage 41 muscle, localized accumulations of the enzyme were obvious in Karnovsky-Roots stained muscle at stage 44. Also, anticholinesterase treatment became effective in prolonging the duration of synaptic currents. MEPC decays at stages 43-59 were approximately doubled by blocking AChE activity with MSF (Table 2). However, even after MSF treatment, the decays at intermediate stages were significantly faster than those recorded at stage 41, which implies that the development of AChE is not the only explanation for the change in decay constant during the early period of synapse development.

Part of the early decline in MEPC duration could be due to a change in the junctional AChR channels, such as a decreased gating time or an increased relative number of fast channels. Evidence regarding these possibilities was obtained from MEPCs with double exponential decays (Fig. 21). Such decays, which may have been due to two classes of receptors with different mean open times, were observed in about 15% of the MEPCs recorded during the 6 week period following stage 43. The mean fast and slow time constants were 0.6 and 2.8 ms (Table 2). Because AChE was abundant during this time, we take the decay constants as upper limit estimates of the channel open times of two kinetic classes of receptors. Following block of AChE, the fast and slow decay constants were comparable to those recorded at stage 41 (Table 2). This result suggests that the gating times of the two classes of channels were unchanged during the early phase of development and indirectly supports the alternative possibility that the fast channels became more abundant.

Following the 6 week plateau (stages 43 to 59), there began a gradual increase in the decay constants of single exponential MEPCs (Fig. 19) which continued through late metamorphosis (stage 64). The values recorded in 2 year old adult superior oblique were not significantly different from those recorded from tadpoles in late metamorphosis. The mean decay constant at stages 64 to adult was 1.7 ms (Table 2) and the distribution of values was skewed (Fig. 20). This gradual increase in decay constant was probably not due to a decline in the activity of junctional AChE, since application of MSF at stages 64-adult lengthened the decay constants of MEPCs to 4.8 ms (Table 2). If the increase in MEPC duration following stage 59 had been due to a decline in AChE, then MSF treatment at late stages should have had little or no effect on the decay constants of

MEPCs. At most, they should have been lengthened to slightly more than 2 ms which would have been comparable to the values recorded in stage 43-59 muscle after block of AChE (Table 2).

Double exponential decays were apparent in about 12% of the MEPCs recorded at stage 64 to adulthood (Fig. 21). The analysis of these MEPCs suggests that the lengthening of MEPC duration may have been due in part to a longer gating time of the slow class of AChR channels. The slow decay constants increased by about 40% from stage 59 through metamorphosis, while there was no significant change in the fast component (Table 2). A similar selective change in the gating time of slow channels has also been seen in developing *Xenopus* myotomal muscle *in vitro* (Leonard *et al.*, 1984) and *in vivo* (see above), although in myotomal muscle the slow channels became faster with age.

At any given stage in the development of the superior oblique, the distribution of decay constants (Fig. 20) reflected a substantial variation *between* recording sites in addition to the variability *within* single sites. The decay constants were narrowly distributed at individual recording sites relative to the variation from site to site, and an analysis of variance indicates that the differences between recording sites were significant (Fig. 20, legend). We did not detect any clustering of values, which would have implied the existence a few discrete types of synapses; instead, there seemed to be a continuum of values ranging from slow to fast at different recording sites.

Table 2

MEPC time course in superior oblique

	Stage 41		Stages 43-59		Stages 64-adult	
		n		n		n
Control						
τ (single)	3.3 ± 1.0	18	1.0 ± 0.3	81	1.7 ± 0.7	118
τ (fast)	1.2 ± 0.4	5	0.6 ± 0.2	27	0.7 ± 0.2	39
τ (slow)	4.2 ± 1.3	5	2.8 ± 0.8	27	3.8 ± 1.2	39
Rise time	1.0 ± 0.4	18	0.6 ± 0.1	81	0.7 ± 0.1	118
MSF treated						
τ (single)	3.3 ± 1.0	10	2.2 ± 1.0	78	4.8 ± 2.3	63
τ (fast)	-	-	1.0 ± 0.4	17	1.0 ± 0.4	3
τ (slow)	-	-	3.9 ± 1.0	17	7.1 ± 0.4	3
Rise time	1.1 ± 0.3	10	0.8 ± 0.2	78	1.2 ± 0.3	63

All values are given as mean ± s.d. (ms). Sample sizes (n) refer to the number of recording sites. τ (single) refers to decay constants of single exponential MEPCs. τ (fast) and τ (slow) refer to decay constants of double exponential MEPCs.

Miniature endplate current development in interhyoideus muscle

During larval development, MEPCs recorded in the interhyoideus muscle were typically longer than those recorded in the superior oblique (Fig. 18 and 19). The earliest MEPCs recorded in the interhyoideus muscle at stage 41 had a mean decay constant of 5.9 ms, almost twice that of the superior oblique (Tables 2 and 3). We observed no double exponential decays, which suggests that most or all of the channels had similar gating times. Unlike the superior oblique, there appeared to be active AChE at the newly formed interhyoideus endplates. Application of MSF lengthened the MEPC durations by about 50% (Table 3). Despite that fact, we did not detect any focal accumulations of AChE after Karnovsky-Roots staining, although a diffuse background stain was evident. A similar observation has been reported at the newly formed endplates of rat diaphragm (Ziskind-Conhaim *et al.*, 1984). The fact that the earliest endplate currents decayed more slowly in the interhyoideus than in the superior oblique suggests that the AChR channel open time was longer in the interhyoideus than the superior oblique.

Although MEPCs in interhyoideus were longer than those in superior oblique, the early schedule of change in the two muscles occurred in parallel (Fig. 19). Within about 16 hours after the first recorded spontaneous synaptic activity, MEPC durations had stabilized at a briefer time course and remained more or less unchanged during the following 7 weeks (stages 44 to 64). During this intermediate period of development the mean decay constant was 2.6 ms (Table 3).

Part of the early decrease in MEPC duration may have been due to a further increase in AChE activity. By stage 44, localized accumulations of AChE were revealed by Karnovsky-Roots stain, and MSF treatment had a greater effect on the time course of MEPCs (Table 3). However, the decay constants were less than those recorded at stage 41, after identical treatment with MSF. We therefore conclude that AChE development does not account for all of the change in MEPC duration during the developmental period from stage 41 to stage 44.

MEPCs with double exponential decays were recorded at stages 44-64 (Fig. 21), whereas they were not present at stage 41. The emergence of a second population of AChR channels with faster gating kinetics could explain the occurrence of double exponential decays and could also account for part of the decline in MEPC duration between stages 41 and 44. Double exponential decays were evident in 9% of the MEPCs recorded at intermediate stages and their mean fast and slow components were 1.1 and 4.0 ms (Table 3). Because AChE was abundant, we use these values as upper limit estimates of the open times of two classes of AChR channels.

The mean fast and slow decay constants of double exponential MEPCs in the interhyoideus were 1.7- and 1.4-fold longer, respectively, than those recorded in the superior oblique at intermediate developmental stages (Tables 2 and 3). This difference cannot be attributed to relatively lower AChE activity in interhyoideus, because it persisted after MSF treatment. Rather, it raises a possibility that channels in interhyoideus had longer gating times than those in the superior oblique.

The differences in channel gating time suggested above are not great enough to account for the difference in single exponential MEPC decays between the two muscles. Differing levels of AChE activity are not a likely explanation either, since MSF treatment had nearly identical effects on the decay times in the two muscles: a 2.4 fold increase in the interhyoideus and a 2.2 fold increase in the superior oblique (Tables 2 & 3). A likely alternative is that the longer MEPC durations in the interhyoideus resulted from a relatively greater proportion of slow AChR channels.

In support of this idea, single channel recordings from extrajunctional regions of these two muscles demonstrate a difference in predominance of channel types (Hartig, Owens, & Kullberg, unpublished). At intermediate stages of development the interhyoideus contains almost exclusively low conductance channels, whereas both high and low conductance channels are observed in the superior oblique. This suggests that the fast and slow time constants of double exponential MEPCs in the interhyoideus may largely reflect contributions from two kinetic classes of low conductance channels, while those from the superior oblique arise from both conductance classes. If low conductance channels are responsible for slow components in both muscles, we would expect their open time in the superior oblique to be substantially briefer than those which contribute to MEPC decay in the interhyoideus.

Beginning in late metamorphosis (about stage 64), the interhyoideus endplates entered another phase of development during which the durations of MEPCs declined markedly (Fig. 19). In adult interhyoideus muscle, MEPCs decayed as single exponentials with a mean time constant of 1.1 ms (Table 3). This value is comparable to the open time of the fast class of AChR channels, as estimated from double exponential decays in stages 44-64. After metamorphosis, no double exponential decays were detected, which suggests that the MEPCs arose from a more or less homogeneous class of fast AChR channels. In comparing the histograms of decay constants at different developmental stages in interhyoideus, it appears that the slower MEPCs dropped out, leaving behind a single population of decay constants centered about 1 ms (Fig. 20). This change in MEPC duration was probably not due to additional cholinesterase development, since complete block of AChE in adult interhyoideus did not produce MEPCs as long as those recorded in stages 44-64 with AChE blocked (Table 3).

As in the superior oblique, the distribution of decay constants in the interhyoideus resulted from significant differences between recording sites as well as variability within single sites (Fig. 20, legend). No clustering of values, indicative of a small number of types of synapses, was evident.

Table 3

MEPC time course in interhyoideus

	Stage 41		Stages 44-64		Adult	
		n		n		n
Control						
τ (single)	5.9 ± 2.0	6	2.6 ± 1.0	151	1.1 ± 0.4	32
τ (fast)	-	-	1.0 ± 0.4	56	-	-
τ (slow)	-	-	4.0 ± 1.3	56	-	-
Rise time	2.0 ± 1.0	6	0.9 ± 0.2	56	0.7 ± 0.2	32
MSF treated						
τ (single)	9.5 ± 3.1	16	6.2 ± 2.8	113	3.9 ± 2.3	43
τ (fast)	-	-	1.8 ± 0.8	5	-	-
τ (slow)	-	-	7.5 ± 1.5	5	-	-
Rise time	3.4 ± 0.8	16	1.7 ± 0.7	113	1.3 ± 0.5	43

See legend to Table 2.

Muscle contraction speed

In stage 50 tadpoles, direct stimulation of the superior oblique or the interhyoideus by extracellular electrodes evoked twitches as well as slower contractions. In order to compare the speed of contraction in the two muscles, we estimated by eye the maximum frequency at which repetitive twitches could be evoked. The maximum twitch frequency in the superior oblique was 28-30 Hz. At higher frequencies, the twitches fused into a sustained tetanic contraction. Single twitches were visibly slower in the interhyoideus than in the extraocular muscle and the maximum twitch frequency ranged from 10 to 16 Hz. Twitches in both muscles, particularly the interhyoideus, were superimposed on slower contractions. With increasing stimulus frequency, it appeared that some fibers reached tetany well before the fastest fibers did. Identical behavior was observed when the muscles were indirectly stimulated via the trochlear or hyomandibularis nerves. Application of TTX (2 μg/ml) abolished propagated twitches, however local contractions could still be produced at the site of contact of the stimulating electrode with the muscle. In the adult interhyoideus, the maximum twitch frequency was increased to 20-24 Hz, and the slower contractions observed in the tadpole were usually absent. In the adult superior oblique, the maximum twitch frequency ranged from 18 to 24 Hz,

which was somewhat slower than in the tadpole. It therefore appears that the contraction speeds of both muscles change during the development from larval stages to adulthood.

Summary of results

1. The development of miniature endplate currents was studied in the superior oblique and interhyoideus muscles of *Xenopus laevis*. An analysis of MEPC decays shows that each muscle possesses its own characteristic program of endplate current development.

2. In the superior oblique, the exponential decay constants of MEPCs were initially about 3 ms; they declined within half a day to 1 ms and remained at that value for 6 weeks. They then gradually became longer, reaching a mean value of 1.7 ms at late metamorphosis.

3. In the interhyoideus, MEPC decay constants were initially about 6 ms. They declined in less than one day to a mean value of 2.6 ms and remained there for the following 7 weeks. Upon completion of metamorphosis, the decay constants underwent a further decrease to about 1 ms.

4. In both muscles, the changes in MEPC decays were correlated with developmental changes in muscle contraction speeds, as measured by maximum twitch frequencies.

5. The above changes in endplate currents in the superior oblique and interhyoideus muscles suggest different programs for the development of AChR channel gating and AChE activity in these two muscles.

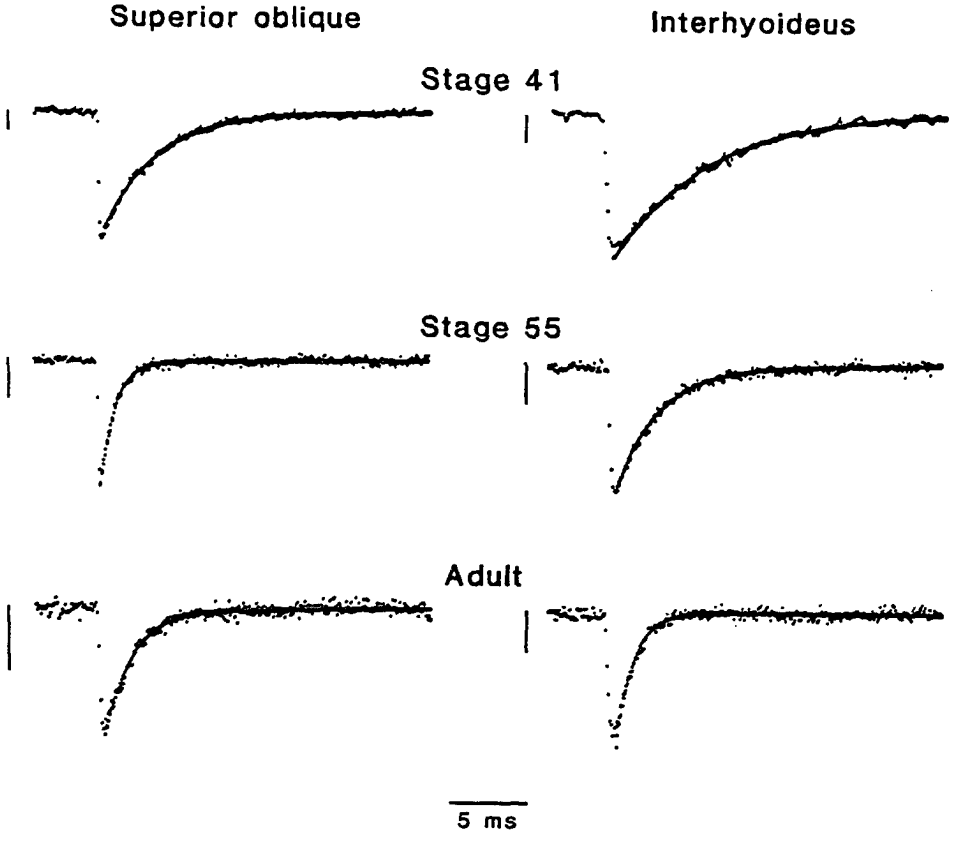

Figure 18. Examples of extracellular MEPCs recorded at different developmental stages. The best-fitting single exponential functions overlap each trace. The decay constants of MEPCs recorded from the superior oblique were 3.4 ms (stage 41), 1.0 ms (stage 55), and 1.8 ms (adult). The decay constants of interhyoideus MEPCs were 6.2 ms (stage 41), 2.7 ms (stage 55) and 1.1 ms (adult). Vertical bars indicate 0.1 mV.

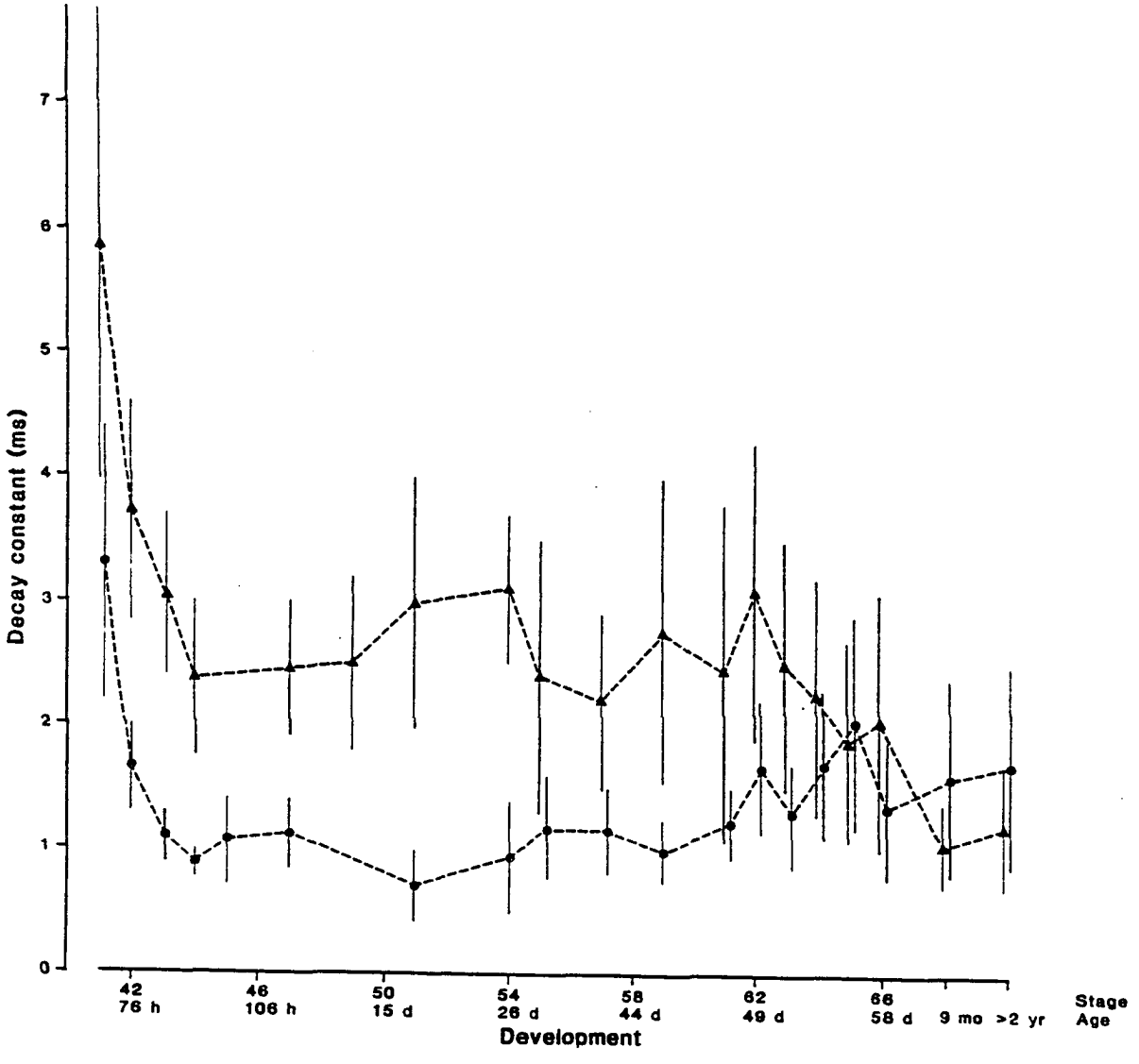

Figure 19. Mean decay constants of single exponential MEPCs recorded at different developmental stages. Bars indicate standard deviations. Each symbol represents the mean decay constant obtained from all recording sites at a single stage of development. The average number of recording sites at each stage was 13 (range: 2 to 38). Circles = superior oblique; triangles = interhyoideus.

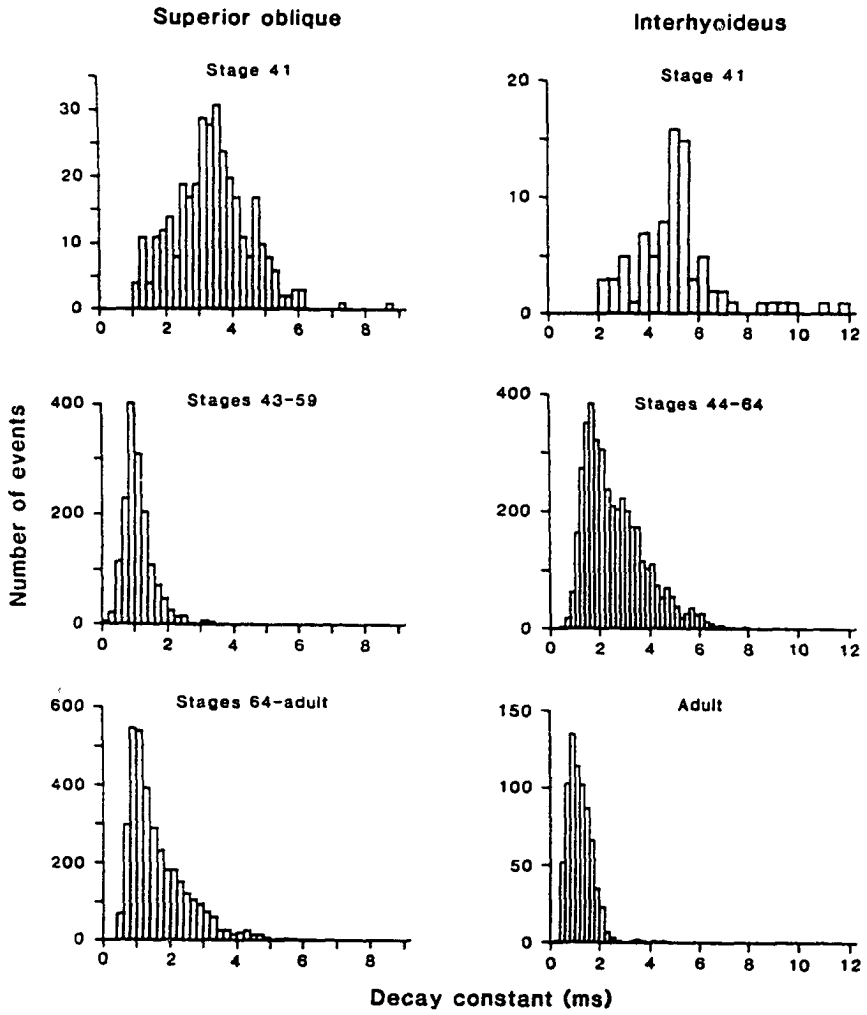

Figure 20. Histograms of MEPC decay constants at different developmental stages. Each histogram is composed of all the MEPC decay constants measured at the indicated stages. A value of F, defined as the ratio of the variance of decay constants *between* sites to the variance *within* sites was calculated for each histogram. The F ratio, the MEPC sample size (n), and the number of recording sites (k) for each histogram are as follows. Superior oblique: stage 41, F=8.55, n=340, k=18; stages 43-59, F=16.0, n=1591, k=82; stages 64-adult, F=64.2, n=3515, k=119. Interhyoideus: stage 41, F=8.6, n=83, k=6; stages 44-64, F=55.0, n=4127, k=151; adult, F=33.4, n=735, k=32. For all histograms, the F ratios are significant beyond the 1% level, indicating that differences between recording sites contributed to the distribution of decay constants.

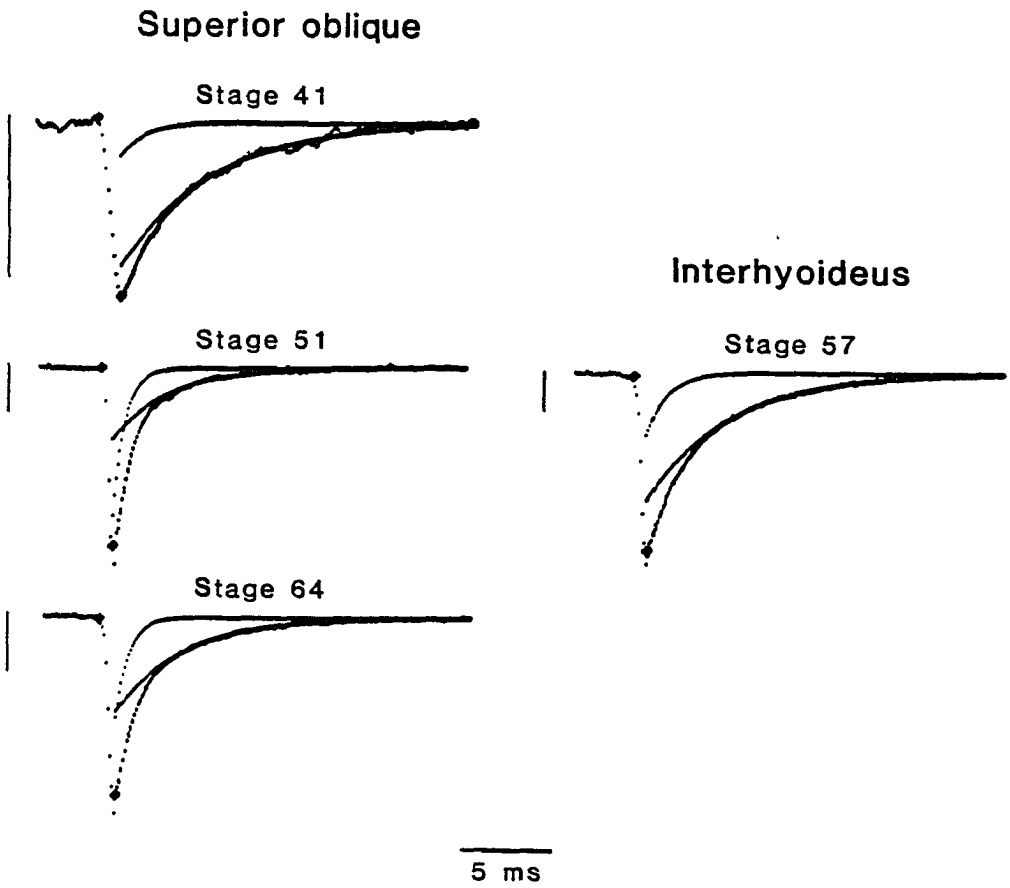

Figure 21: Double exponential MEPCs recorded at endplates of superior oblique and interhyoideus muscles at different developmental stages. The fast and slow exponential components are shown as well as their sums, which in all cases closely overlap the data points. In the sample MEPCs from superior oblique endplates, the fast and slow decay constants were 1.4 and 4.0 ms (stage 41), 0.7 and 2.9 ms (stage 51) and 0.7 and 3.6 ms (stage 64). The fast and slow decay constants in the sample MEPC from stage 57 interhyoideus were 1.2 and 4.5 ms. Vertical bars indicate 0.1 mV.

DISCUSSION

Development of Acetylcholine Receptors

The single channel properties of AChRs on myotomal muscle were studied from the time of their first appearance on the muscle until just before resorption of the muscle in metamorphosis. Myotomal muscle is the first muscle in *Xenopus* embryos to develop. It becomes recognizable ~19 hr after fertilization. The first AChR activity we could detect was at stage 19 (21 h old) which is when AChRs can first be detected by uptake of ^{125}I-α-BTX (Chow & Cohen, 1983).

During the first 14 h (stage 19-30) after the initial appearance of AChRs, the membrane was dominated by channels with long open times (~3 ms) and low conductances (30-40 pS). Noteworthy changes in channel function began less than two days after fertilization (~stage 33/34). At this time, a class of large conductance channels (45-60 pS) with brief open times (0.8 ms) was observed in almost all recordings, and as development progressed, these channels became the dominant ones on the myotomal muscle. Over the range of development during which large channels were abundant enough for analysis (stages 33 to 61), their conductance and gating properties remained constant. We observed no change in the conductance of the small channel during development but found that the open time of the small channel became about 50% briefer and remained so from then on. The reduction in small channel open time coincided with the appearance of significant large channel activity, suggesting possible coregulation of these events. A previous study of channel open time during development of the myotomal muscle was done by spectral analysis of ACh induced noise (Kullberg & Kasprzak, 1985). There is good agreement between the present study and the noise study with regard to the schedule of change in channel open time, but the noise study did not give any information about conductance or the change in gating kinetics of the low conductance class of receptors.

As previously observed in myotomal muscle (Brehm *et al.*, 1984a), the current-voltage relationship of both channels was nonlinear. Over a hyperpolarizing range of 0 to 80 mV, the channel conductance increased linearly with applied potential. At rest, the slope conductances were 33 and 43 pS, while at 40 mV applied potential, the mean slope conductances were 40 and 57 pS. This relationship between conductance and voltage has not been reported for the ACh receptors in mammalian muscle (Vincini & Schuetze, 1985; Siegelbaum *et al.*, 1984). The basis for this difference is not known.

There has not been a detailed study of the *in vivo* development of AChR single channel properties in any other species. However, the data presently available suggest that there are some fundamental similarities as well as differences between *Xenopus* and other

vertebrates. The development of channel properties in rat is generally similar to that in *Xenopus* except that the conversion from slow to fast gating channels occurs over a longer time course in rat muscle (Vincini & Schuetze, 1985). The bovine ACh receptor is also reported to have a lower conductance and a longer open time in embryonic muscle than in the adult muscle (Mishina *et al.*, 1986). There is no indication that a change in gating kinetics of the low conductance class of receptors occurs in mammalian muscle (Schuetze & Vincini, 1986). The only report of ACh receptor development in the chick indicates that the channel open time does not become briefer during muscle maturation (Schuetze, 1980). No other avian muscles have been investigated.

Mechanism of change in acetylcholine receptor properties

Serum from patients with myasthenia gravis contains antibodies which bind to embryonic AChRs in rat. Studies with these antibodies indicate that there is a change in the antigenic properties of ACh receptors which is correlated with a developmental change in channel open time (Hall *et al.*, 1985). The myasthenic antibodies selectively block slowly gating channels (Schuetze *et al.*, 1985). The nature of the differences detected by the antibodies is not known, however it may be related to changes in the subunit composition of the AChR during development. Mishina *et al.* (1986) found that a change from low to high conductance with associated gating changes occurs when the γ subunit of the bovine AChR is replaced with the ϵ subunit. It is not known if subunit substitution occurs in other species as well, but Brehm *et al.* (1987) found that inhibiting protein synthesis prevented appearance of high conductance channels in cultured *Xenopus* myotomal muscle.

In addition to this mechanism of change, there is indirect evidence that modification of existing channels may also be important during development of channel function. Work by Vicini & Schuetze (1986) in developing rat muscle supports the idea that alterations in receptor properties may occur *in situ*. They measured MEPC decay over extended intervals at individual developing rat endplates and found that fast channel activity develops more rapidly than would be expected from the rate of receptor turnover.

No mechanism for the decrease in open time of the low conductance receptor has been proposed, but it does not appear to depend on glycosylation or protein synthesis (Carlson *et al.*, 1985).

Comparison of acetylcholine receptor development in vitro and in vivo

It is difficult to compare the schedule of AChR development *in vivo* and *in vitro* due to the lack of criteria for staging in the latter. High conductance channels are the dominant channel type in cultured muscle after 5 days (Leonard *et al.*, 1984; Brehm *et al.*, 1984),

but whether the onset of fast channel activity occurs as a synchronized event in cells corresponding in age to stage 33 is not known. Studies of AChR development in aneural cell culture from *Xenopus* myotomal muscle differ with regard to the change in gating kinetics of the 40 pS channel. Brehm *et al.* (1984b) saw no difference in open time of the small channels in one day vs. five day old cultures. In contrast Leonard *et al.* (1984) found that the low conductance channel underwent a three fold reduction in open time within a similar period. This discrepancy has not been resolved but may be due to differences in the age of muscle cells at the time of recording. Both groups dissected and plated myotomal muscle cells from embryos before the onset of acetylcholine sensitivity; however, Leonard *et al.* (1984) limited their early stage recordings to a period corresponding to about stage 21-24 (~ 5 h), while Brehm *et al.* considered early stage records to be those taken over a 24 h period after plating the cells.

Temporal relation to other features of neuromuscular development

Developmental events occurring simultaneously are often functionally related and may be mutually regulated (Merlie & Sanes, 1986). Therefore it is interesting to examine the temporal relationship between changes in receptor function and other processes in neuromuscular development. The most dramatic change in channel function occurs at about stage 33 when 60 pS channels emerge in increasingly large numbers, and 40 pS channels develop faster gating times. These events occur just a few hours prior to the onset of hatching and free swimming.

AChE also becomes abundant at the synapse just prior to hatching. Although channel gating undergoes little change during the first day of synaptogenesis, there is a considerable decrease in the duration of endplate currents during that time. This is due to the deposition of AChE at the developing synapse. Endplate currents first become rate-limited by channel closing at about stage 33, which reflects a mature level of AChE activity. There is evidence that AChRs and AChE are mutually transported to the membrane (Porter-Jordan *et al.*, 1986) and their expression may be coordinately regulated (Merlie *et al.*, 1986).

Electrical coupling (Armstrong *et al.*, 1983) and its morphological correlate, the gap junction (Kullberg *et al.*, 1977), are extensive in myotomal muscle prior to hatching. There follows a progressive loss of coupling with muscle maturation which parallels the increased expression of large conductance channels on nonjunctional membrane. These processes may be coregulated during myogenesis, although they differ with respect to regulation by muscle activity. Immobilization greatly retards the normal uncoupling of muscle cells (Armstrong *et al.*, 1983) but has no effect on development of fast gating channels, as deduced from synaptic currents (see Results).

In mature muscle there is a direct correlation between the contraction speed of the muscle and channel open time. Muscles which contract slowly have AChR channels with long open times, whereas the channel open times are brief in fast twitch muscles (Miledi & Uchitel, 1981; Foderov *et al.*, 1982). Channel open time is similarily related to muscle contraction speed during development. In rat, muscles which are destined to become fast in the adult contract more slowly in the neonate (Close, 1964). Correlated with this change in contraction speed is a sequential expression of three myosin heavy chain isozymes during muscle development (Whalen *et al.*, 1981). No comparable data on the development of the contractile apparatus in myotomal muscle is available, but early in development, when slowly gating channels dominate the membrane, muscle contraction is visibly slow. As development proceeds, both channel gating and contraction speed become more rapid. These observations suggest that channel gating is matched to the contraction speed of developing muscles. Further support for this idea comes from the study of developing endplate currents in other *Xenopus* muscles (see below).

During the transition from low to high conductance channels a second set of motor neurons emerges from the larval spinal cord (Nordlander, 1986) and eventually replaces most of the primary motor neurons (Hughs, 1959; Forehand & Farel, 1982). There is no direct experimental data to suggest that the two types of neurons affect receptor properties differently. However, when embryonic neurons were added to cultured *Xenopus* myocytes which had already undergone receptor development, the channel open times unexpectedly became longer (Brehm *et al.*, 1982).

Functional significance of different acetylcholine receptor channel types

The physiological significance of expressing more than one channel type during development is not understood. One possibility is that embryonic channels provide a larger signal in response to neurotransmitter than do adult channels. Receptor density at the site of nerve-muscle contact is initially low. Embryonic channels pass approximately twice the charge per opening as do adult channels, and may thereby compensate for the lower density. However, the need for synaptic potentials at the very earliest stages of synapse development (Blackshaw & Warner, 1976a; Kullberg *et al.*, 1977) in myotomal muscle is not clear, since the muscle is not yet capable of contraction. Possibly the earliest synaptic currents serve functions which are unrelated to muscle contraction. For instance, entry of calcium ions through the receptor channel might provide developmental cues related to the state of innervation or may promote receptor clustering at the synapse (Peng, 1984).

Replacement of slow gating channels with faster gating ones during development may be necessary to limit calcium influx through the endplate (Schuetze and Role, 1987). Calcium-induced myopathy at the endplate region has been reported in muscle when synaptic currents were prolonged by blocking AChE activity (Salpeter *et al.*, 1982) and also in human muscles with abnormally slow gating AChRs (Engle *et al.*, 1982). Increased calcium influx through embryonic receptor channels may be advantageous during myogenesis, however. In developing insect muscle the calcium sequestering and releasing mechanisms mature after muscle contraction begins in the embryo (Tyrer, 1973). Likewise, in myotomal muscle cytoplasmic ultrastructure is relatively undifferentiated at the time movement begins (Kullberg *et al.*, 1977). Calcium entering through the endplate channels may supplement that which is released from the developing sarcoplasmic reticulum and may thereby help to initiate muscle contraction in the embryonic muscle.

It may be misleading to consider slowly gating channels as embryonic. Evidence from adult amphibian slow muscle (Miledi & Uchitel, 1981, Foderov *et al.*, 1982) and from the slowly contracting interhyoideus in developing *Xenopus* (see Results) demonstrates that slow channels can be abundant in mature muscle. One possible advantage of expressing more than one kinetic class of ACh receptors may be to allow muscle to tailor synaptic current duration to other functional characteristics, such as contraction speed. With more than one channel type, a muscle could regulate the duration of its synaptic currents by altering the relative numbers of fast and slow channels at the synapse. Slowly contracting muscles such as the classical slow amphibian muscle fibers, which do not generate action potentials and have a widely distributed innervation, would benefit from having channels which pass charge over a prolonged period of time. Channels with a long open time could impede the function of fast twitch fibers which need to generate rapid, repeated contractions. This hypothesis suggests that ACh receptor properties may be mutually regulated with other muscle proteins, such as myosin ATPase and voltage gated channels, which give muscles their distinctive slow or fast contractile properties. As a parallel to the existence of two AChR channel types, it is reported that muscle fibers may have different mixes of slow and fast myosin ATPase (see review by Miller & Stockdale, 1987).

Recent studies have complicated our estimate of the number of functionally distinct forms of AChR present in muscle. There is increasing evidence for more than one kinetic form of low conductance channel in *Xenopus* and in mammalian muscle (Steele & Steinbach, 1986). Denervation of mature muscle is accompanied by incorporation of a second class of receptor with low conductance in nonjunctional membrane of frog muscle (Allen & Albuquerque, 1985) and in both junctional and nonjunctional membranes of the mouse (Henderson *et al.*, 1987). Auerbach & Lingle (1986) have found that both the high and low conductance classes in *Xenopus* have multiple burst

modes which might reflect different kinds of channels. In addition, there may be two classes of AChRs with smaller conductances that those described here. The full diversity of AChR channels in skeletal muscle is not yet known, nor do we understand either the functional significance or the molecular basis for this diversity.

Development of Endplate Currents in Immobilized Muscle

To determine whether motor activity is a requirement for the normal development of synaptic currents in myotomal muscle, movement was eliminated in developing embryos by removing their egg membranes and placing them in a solution of tank water and TTX. By this procedure motor activity was blocked without surgical intervention or the use of anesthetics, which in some cases may alter ACh receptor kinetics (e.g., Katz & Miledi, 1975; Ruff, 1977; Neher & Steinbach, 1978; Ogden *et al.*, 1981)

Our data show that the developmental changes in rise times and decay rates of endplate currents are not dependent on mechanical activity of muscle nor presumably on TTX-blockable action potentials in either nerve or muscle. Of the many factors which may influence the shape of the MEPC (reviewed by Gage, 1976; Steinbach & Stevens, 1976), two important factors are the gating time of ACh receptor channels and the rate of hydrolysis of transmitter by AChE (Anderson & Steven, 1973; Wathey *et al.*, 1979). Our results therefore suggest that the deposition of junctional AChE and the change in gating kinetics of ACh receptor channels during development are not affected by the absence of motor activity.

Development of acetylcholinesterase activity

As a test of the implication that AChE activity develops normally in immobilized muscle, we examined the effects of blocking AChE activity in 2 and 5 day old animals. MEPCs in immobilized and control muscle were prolonged similarly by treatment with an irreversible anticholinesterase (MSF), indicating that AChE limits the duration of transmitter action similarly in both kinds of muscle. In contrast to this observation, block of electromechanical activity in developing chick muscle has been shown to reduce the endplate-specific form of AChE (Rubin *et al.*, 1980; Betz *et al.*, 1980) and to result in longer synaptic currents (Rubin *et al.*, 1980). Electromechanical activity of muscle is also required for normal levels of AChE activity to be achieved at developing ectopic synapses in rat skeletal muscle (Lomo & Slater, 1980; Cangiano *et al.* 1980). Harris (1981), however, reports that lack of muscle activity in rat embryos developing *in utero* did not affect the development of junctional AChE, which suggests that synapses in embryonic rat muscle may differ from ectopic synapses in mature muscle with respect to the control of AChE by electromechanical activity. Sohal (1984) also reports that the high molecular weight form of AChE developed in both paralyzed and aneural

extraocular muscle of duck embryos. In developing *Xenopus* muscle, Cohen *et al.* (1984) demonstrated that AChE activity increased despite immobilization by tricaine, a local anesthetic. However, MEPC durations in immobilized animals did not become as brief as those in control animals, and it was suggested that either AChE development was retarded or some other determinant of MEPC time course was affected by tricaine treatment. Our results indicate that any such effects of tricaine must be unrelated to immobilization.

Development of acetylcholine receptor channel gating

In addition to suggesting that AChE developed normally in immobilized muscle, the similarity of MEPC decays in control and TTX-treated muscle before and after block of AChE suggests that ACh receptor gating time was unaffected by immobilization. If substantial differences in channel gating time had resulted from the TTX treatment, there should have been dissimilarities in the decay phases of MEPCs in both the presence and absence of AChE activity (Magleby & Stevens, 1972; Anderson & Stevens, 1973). Both single and double exponential decays were comparable in the two rearing conditions. The time constants of the fast and slow components of double exponential decays were about 0.7 and 3.0 ms in the oldest muscle studied. However, in both immobilized and control animals we noticed that both the fast and slow decay constants were considerably longer at the time of their first appearance than at later stages (Fig. 5). This could reflect an incomplete development of AChE when the earliest double exponentials MEPCs were recorded. Another possible explanation is that when double exponential MEPCs first appear the two components mostly reflect contributions from the fast and slow gating low conductance channels whose apparent open times are 1.6 and 2.8 ms as measured in single channel recordings. Later in development when both components become briefer the fast component may be due to high conductance channel activity (apparent open time = 0.8 ms) and the slow component to fast gating low conductance channels. This idea is consistent with the changing ratio of channel types we found in single channel recordings during development. The slow components of the MEPCs are slightly longer in duration than the apparent open time of the two kinetic classes of low conductance channels. The difference may reflect bursting activity, which would prolong the apparent open time as found in spectral analysis of ACh noise and may also prolong the decay of synaptic current.

In vitro studies of *Xenopus* muscle support our conclusion that muscle activity is not required for the change in gating time of ACh receptors. Both single channel recordings (Brehm *et al.*, 1984b) and spectra of ACh-induced membrane noise (Brehm *et al.*, 1982) show that the frequency of fast channel openings relative to slow openings increases with age in noncontracting, aneural cultures of muscle. In species other than *Xenopus laevis* there is no published evidence bearing directly on the role of electromechanical

activity in the control of ACh receptor kinetics. In developing chick muscle there is no change in ACh receptor gating time (Schuetze, 1980), so it seems unlikely that muscle activity should affect receptor kinetics. In developing rat muscle it appears that the conversion to fast kinetics will not occur if the muscle is denervated at birth (Schuetze & Vicini, 1983). On the other hand, Brenner, Meier & Widmer (1983) found that at ectopic synapses in adult rat muscle, only temporary contact with a foreign nerve is required to induce subsequent changes in gating time. In their study, however, the muscle was kept active by direct stimulation in order to reduce nonjunctional receptor levels. The possibility that muscle activity regulates the gating time of ACh receptor channels in rat muscle remains to be tested.

Comparative Development of Endplate Currents

The comparison of endplate currents in developing interhyoideus and superior oblique muscles demonstrates that endplates of different muscles may vary in their programs of physiological development and that more than one distinct phase of development can occur within a single muscle. In other studies of developing endplates in vertebrate skeletal muscle changes in synaptic currents have been shown to be due primarily to the development of AChR channel kinetics and AChE activity (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1980; Michler & Sakmann, 1980; Kullberg *et al.* 1980; Kullberg & Kasprzak, 1985; Vicini & Schuetze, 1985). It is likely that these two factors can account for most or all of the developmental changes in synaptic current duration which we have seen in the superior oblique and interhyoideus muscles of *Xenopus*. Our results suggest a specific program, which we summarize below, for the development of endplate function in each muscle.

Superior Oblique:

At newly formed endplates in superior oblique there is negligible AChE activity. The enzyme accumulates rapidly and, within half a day after the first detectable synaptic activity, the decay times of endplate currents decrease 3-fold. The AChR channels present at the onset of synaptic activity have predominantly brief open times (less than 1 ms). A second, less numerous, class of channels with slower gating times is also present. During the following half day of development, there is an increased relative amount of fast channel activity, with no change in the gating time of either class of channels. The estimated mean open times of the two classes of channels are 0.6 and 2.8 ms. After the initial decline in MEPC duration, there follows a 6 week period during which no change occurs in the properties of AChR channels or AChE activity. At about the time of metamorphosis, endplate currents gradually become slower, possibly due to a lengthened gating time of the slow class of receptors. The adult state of the endplates is reached shortly before the completion of metamorphosis, and there is no further change in AChE activity or channel properties in the subsequent 2 years of adulthood.

Interhyoideus:
At the time of first detectable synaptic activity, the interhyoideus differs from the superior oblique in two respects: AChE activity is already evident, and the gating times of AChR channels are predominantly or exclusively slow. During the following day, there is further development of AChE activity and an emergence of fast AChR channel activity. After the initial decline in MEPC duration, there is a 7 week period during which no further change occurs in channel properties or AChE activity. MEPC decays are 2.6-fold longer on the average than those in the superior oblique, due to a predominance of slow AChR channels. Both fast and slow channels are active and their estimated gating times are 1.0 and 4.0 ms, somewhat longer than those in the superior oblique at the same stages. Following metamorphosis the durations of endplate currents decline due to a loss of slow channel activity. At adult interhyoideus endplates AChE is abundant, the predominant channels have an open time of about 1 ms and there is little or no slow channel activity.

The schemes proposed above are based on the analysis of changes in MEPC decays. In order to study the development of AChE, we have used an irreversible anti-cholinesterase, MSF, under conditions which should entirely block the enzyme activity. Any developmental changes in endplate current decay which persisted after MSF treatment we have tentatively attributed to changes in the properties of AChRs, either in their gating times or in the relative numbers of slow and fast receptors. We have not directly measured AChR channel gating times, but have assumed that the endplate current decay constant sets an upper limit estimate of the mean channel open time at endplates which have abundant AChE (Anderson & Stevens, 1973). The true open time will tend to be overestimated by this procedure, due to the bursting behavior of channels (Colquhoun & Sakmann, 1981; Dionne & Leibowitz, 1982). Also, even when AChE is fully active, the decay of endplate currents at adult frog neuromuscular junctions (at 21° C) is reported to be slower than the channel open time, due to the rebinding of ACh to receptors (Feltz, Large & Trautmann, 1977). Our conclusions about the development of channel gating at the synapse therefore remain to be confirmed by direct recordings of channel open times.

The likelihood that each muscle expresses two discrete kinetic classes of AChRs is argued by the presence of double exponential decays in both muscles. Similar double exponential MEPCs have been recorded in rat skeletal muscle (Sakmann & Brenner, 1978; Fischbach & Schuetze, 1978; Michler & Sakmann, 1980; Vicini & Schuetze, 1985) and *Xenopus* myotomal muscle (Kullberg & Kasprzak, 1985; and see above), and in those cases the fast and slow decay constants have been shown to resemble the mean open times of two classes of ACh receptor channels. In this study, we have used the fast and slow components of double exponential decays as estimates of the gating times of classes of AChR channels, at those stages where AChE is abundant. The slow time

constant of MEPCs from the interhyoideus was substantially longer than that from the superior oblique both before and after block of AChE. Channels responsible for the slow component in the two muscles may differ in their gating properties, with those in the superior oblique having briefer open time than those in the interhyoideus. Two kinetic classes of low conductance channel are known to exist in *Xenopus* muscle (Leonard *et al.*, 1984; see above), both of which have longer open times than the high conductance channel. A differential distribution of channel types in the superior oblique and interhyoideus is probably responsible for the differences we observed in MEPC decays. The fact that most MEPC decays were apparently well fitted by single exponential curves probably reflects our inability to resolve two components by eye when one component largely outweighs the other.

The duration of endplate currents was found to be correlated with muscle contraction speed, as measured by maximum twitch frequencies. The slowest endplate currents and contraction speeds were observed in the larval interhyoideus, whereas the fastest of each was present in the larval superior oblique. During metamorphosis the interhoideus developed faster contraction speed and faster endplate currents, while both slowed in the superior oblique. If our explanation of the change in MEPC decays according to channel properties is correct, these results suggest that the ratio of channel types and/or their gating properties is regulated mutually with the contraction speed of the twitch muscle fibers. It would follow that the distribution of MEPC durations which we have observed within each muscle may correspond to a variety of functionally different fiber types (see review by Miller & Stockdale, 1987).

In both the superior oblique and interhyoideus, there are some obvious changes in muscle function which are correlated with the development of synaptic currents and with changes in contraction speed. The rapid saccadic movements of the tadpole eye cease during metamorphosis and the only obvious movement of the adult eye is a protective retraction. The phasic contractions of the interhyoideus, which are essential to feeding and branchial ventilation in the tadpole, also cease during metamorphosis. In *Rana catesbeiana*, the phasic contractions of the interhyoideus are produced by fibers which have some of the properties of adult slow muscle, such as resistance to fatigue when stimulated directly, sustained contraction in the presence of ACh, and simple endplate morphology (Gradwell & Walcott, 1971). However, these fibers also generate twitches and therefore may be similar to the intermediate type fibers described by Lannergren (1979). The fibers responsible for slow, phasic contractions disappear after metamorphosis in *Rana catesbeiana*, leaving only fibers which have the properties of fast twitch muscle (Gradwell & Walcott, 1971).

We have not determined whether the metamorphic changes of endplate currents occur within individual fibers or whether they represent the appearance of new types of fibers. In *Rana temporaria*, larval interhyoideus fibers are histolyzed and replaced by new, adult fibers during metamorphosis (De Jongh, 1968). However, the presynaptic elements may persist through metamorphosis. A study in *Rana* by Alley & Barnes (1983) indicates that adult fibers in metamorphosing jaw muscles are innervated by the same motorneurons as in the larvae. If a similar process occurs in *Xenopus*, then the change in time course of MEPCs at metamorphosis may be due to the establishment of new endplates on adult muscle fibers, rather than to the further maturation of old endplates.

Conclusion

The studies reported here have given new insights into the development of two crucial elements of postsynaptic function: AChRs and AChE. (1) Details on the character and chronology of AChR channel development have been revealed by single channel studies in developing myotomal muscle. (2) Examination of synaptic currents in immobilized muscle demonstrated that the development of synaptic AChR and AChE function is not regulated by motor activity. (3) On the other hand, functional development of AChRs and AChE may be regulated differently according to muscle function. The comparison of developing synaptic currents in superior oblique and interhyoideus muscles showed that different muscles can have unique programs for the development of postsynaptic functions. In addition to extending our knowledge of synaptogenesis, these studies have laid the foundation for further investigations into the molecular control of postsynaptic function in developing muscle.

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